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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.53(c).

	Docket Number	14028.0295US	+	

INVENTOR(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)
Neville	David	M.	9624 Parkwood Drive, Bethesda, Maryland 20814
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TITLE OF INVENTION (500 characters max)

METHODS FOR EXPRESSION AND PURIFICATION OF IMMUNOTIXINS

CORRESPONDENCE ADDRESS

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PATENT TRADEMARK OFFICE

ENCLOSED APPLICATION PARTS (*Check All That Apply*)

- ☒ Provisional Application Title Page — *Number of Pages* [2]
- ☒ Specification (includes Description, Claims, & Abstract) — *Number of Pages* [83]
- ☒ Drawing(s) — *Number of Sheets* [27]
- ☒ Authorization to Treat Reply Requesting Extension of Time as Incorporating Petition for Extension of Time
- ☒ Other (specify): Sequence Listing and Postcard

METHOD PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*Check One*)	
☐ Applicant claims small entity status. See 37 CFR § 1.27. ☒ A Credit Card Payment Form PTO-2038 is enclosed to cover the filing fees. ☐ A check or money order is enclosed to cover the filing fees. ☐ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number _____. ☐ The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. _____.	**FILING FEE AMOUNT** $ 160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☒ Yes. The name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Signature _____ **Date** August 1, 2003

Typed or Printed Name: Tina Williams McKeon, Ph.D., J.D.

Registration No. 43,791

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

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_____ 8/1/03

Kerry Morgan Date

ATTORNEY DOCKET NO. 14028.0295US
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Neville, et al.	)	Art Unit: Unassigned
	)	
Application No. Unassigned	)	Examiner: Unassigned
	)	
Filing Date: Concurrently	)	Confirmation No. Unassigned
	)	
For: **METHODS FOR EXPRESSION AND PURIFICATION OF IMMUNOTOXINS**	)	

AUTHORIZATION TO TREAT REPLY REQUIRING EXTENSION OF TIME AS INCORPORATING PETITION FOR EXTENSION OF TIME

Mail Stop PROVISIONAL PATENT APPLICATION
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Sir:

Pursuant to 37 C.F.R. § 1.136(a)(3), the Commissioner is hereby requested and authorized to treat any concurrent or future reply in the above-identified application, requiring a petition for an extension of time for its timely submission, as incorporating a petition for extension of time for the appropriate length of time.

ATTORNEY DOCKET NO. 14028.0295US
PATENT

The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

NEEDLE & ROSENBERG, P.C.

Tina Williams McKeon, Ph.D.
Registration No. 43,791

NEEDLE & ROSENBERG, P.C.
Customer No. 23859

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Kerry Morgan — 8/1/03

Kerry Morgan — Date

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UTILITY PATENT

APPLICATION FOR LETTERS PATENT

TO WHOM IT MAY CONCERN:

Be it known that we, David M. Neville, a citizen of the United States of America; Jung-Hee Woo, a citizen of Korea; and Yuan-Yi Liu, a citizen of China, residing, respectively, at 9624 Parkwood Drive, Bethesda, Maryland 20814, 1603 East Jefferson Street, Apartment 201, Rockville, Maryland 20852, and 259 Congressional Lane, Apartment 102, Rockville, MD 20852 have invented certain new and useful improvements in

METHODS FOR EXPRESSION AND PURIFICATION OF IMMUNOTOXINS

of which the following is the specification.

METHODS FOR EXPRESSION AND PURIFICATION OF IMMUNOTOXINS

[0001] The U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates generally to methods of protein expression and purification, and more specifically, to methods of expression and purification of immunotoxins.

Description of the Related Art

[0003] The number of organ transplants performed in the United States each year is approximately 24,000 and consists predominantly of kidney transplants (14,000), liver transplants (5,000), heart transplants (2,200), and smaller numbers of pancreas, lung, heart-lung, and intestinal transplants (2002 OPTN/SRTR Annual Report).

[0004] Transplant tolerance remains an elusive goal for patients and physicians whose ideal would be to see a successful, allogenic organ transplant performed without the need for indefinite, non-specific maintenance immunosuppressive drugs and their attendant side effects. Many of these patients have been treated with cyclosporin, azathioprine, and prednisone with a variety of other immunosuppressive agents being used for induction or maintenance immunosuppression. The average annual cost of maintenance immunosuppressive therapy in the United States is approximately $11,000 (Immunosuppressive Drugs Coverage Act, National Kidney Foundation, available at http://www.kidney.org/general/pubpol/immufact.cfm). While these agents are effective in preventing rejection, the side effects of immunosuppressive therapy are considerable. Immunosuppressive therapy induces nonspecific unresponsiveness of the immune system. Recipients are susceptible to infection and there is a risk of malignancy such as in the form of post transplant lymphoproliferative disorders. A major goal in transplant immunobiology is the development of specific immunologic tolerance to organ

transplants with the potential of freeing patients from the side effects of continuous pharmacologic immunosuppression and its attendant complications and costs.

[0005] A bivalent anti-T cell immunotoxin, A-dmDT390-bisFv(G_4S) was developed for treatment of tolerance induction for transplantation, T-cell leukemia and autoimmune diseases. The immunotoxin consists of the first 390 amino acid residues of diphtheria toxin (DT390) and two antigen-binding domains (sFV) of the anti-CD3ε antibody, UCHT1. The anti-CD3ε antibody moiety enables the immunotoxin to target specific cells and the diphtheria toxin moiety kills the target cells. The A represents an alanine residue present at the amino terminus of the DT domain. The immunotoxin may be utilized to effect at least partial T-cell depletion in order to treat or prevent T-cell mediated diseases or conditions of the immune system. Other types of toxin or antigen-binding moieties may be utilized in anti-T cell immunotoxins and as immunotoxins against other cells as well.

[0006] Administration of an anti-T cell immunotoxin provides an approach for specific immunologic tolerance. It will potentially be of tremendous value and appeal to patients and transplant physicians throughout the world. It is applicable to new organ transplants and potentially to existing transplants in recipients with stable transplant function. The immunotoxin can provide highly specific immunosuppression and imparts transplant tolerance in primates, without the adverse effects of nonspecific immunosuppressive drugs, anti-lymphocyte serum or radiation. It is a goal that the rejection response is inhibited to the point that rejection is not a factor in reducing average life span among transplant recipients.

[0007] The methylotrophic yeast *Pichia pastoris* has been used successfully to express heterologous proteins from different origins (Gellissen 2000). As an eukaryote, *Pichia pastoris* has the ability to perform many post-translational protein modifications such as proteolytic processing, folding, disulfide bond formation and glycosylation. Like other yeasts, *Pichia pastoris* offers significant advantages over higher eukaryotic cells such as Chinese hamster ovary (CHO) or baculovirus-infected insect cell expression systems. It is easy to manipulate, has a rapid growth rate and requires inexpensive media. These greatly reduce the production time and cost, especially on a commercial scale.

Unlike Saccharomyces cerevisiae, *Pichia pastoris* is not a strong fermentor and can be easily cultured to very high cell density of >100 g dry cell weight /liter (Siegel et al., 1989). This, plus the strong AOX1 promoter employed in driving transcription of foreign genes, have made *Pichia pastoris* the system of choice for high levels of expression of heterologous proteins. The AOX1 promoter also has advantages in the expression of foreign proteins that are deleterious to the expressing host because the promoter is tightly regulated and highly repressed under non-methanolic growth conditions. The inducible and tightly regulated AOX1 promoter has allowed successful expression of DT based immunotoxins, in secreted form, in *Pichia pastoris* strains without any mutation to confer a resistance to DT. (Woo et al., 2002). However, diphtheria toxin (DT) is a very potent toxin to all eukaryotic cells if its catalytic domain can find a route to the cytosol. *Pichia pastoris* is inherently sensitive to these toxins.

[0008] *Pichia pastoris* may be grown in a fermentor. One protocol for *Pichia pastoris* fermentation contains glycerol as the initial carbon source, followed by brief carbon starvation and use of methanol as the carbon source (*Pichia pastoris* Fermentation Using a BioFlo 110 Benchtop Fermentor, New Brunswick Scientific). Glycerol is typically fed to the yeast when the dissolved oxygen rises above 40%. When the dissolved oxygen rises again, the feed is switched to methanol. Glycerol is then fed whenever the dissolved oxygen rises above 40% and until the level drops below 40%.

[0009] Woo et al. disclosed that, when expressing a bivalent anti-human anti-T cell immunotoxin A-dmDT390-bisFv(G_4S) in *Pichia pastoris*, a buffered complex medium at pH 7.0 with 1% casamino acids provided the highest expression in shake flask culture and that the expression level was improved by adding PMSF in the range of 1 to 3 mM. (Protein Expression and Purification 25:270-82 (2002)).

[0010] Sreekrishna disclosed that an increased secretion level was obtained using *Pichia pastoris* in shake flask cultures when the cells were highly aerated and in a buffered medium at pH 6.0 that was supplemented with yeast extract and peptone (Chapter 16, Industrial Microorganisms: Basic and Applied Molecular Genetics (1993)). The growth medium contained yeast nitrogen base with ammonium sulfate, biotin and

glycerol buffered to pH 6.0 with potassium phosphate buffer as well as yeast extract and peptone. The induction medium contained methanol in place of glycerol.

[0011] U.S. Patent No. 6,492,498 disclosed expression of dimeric DT_{390} anti-CD3.cys in E. coli. Purification of the expressed protein required centrifuging the bacteria, resuspending the pellet and treating with lysozyme. The pellet was then homogenized and incubated in Triton X-100 buffer. Inclusion bodies were collected by centrifugation, homogenized in Triton X-100 buffer and centrifuged 3 more times. The process was repeated 4 times. The dimeric immunotoxin was obtained by sonication of the fusion protein in the presence of a denaturing and reducing buffer. The protein was purified over a Q-Sepharose column and a TSK GS3000SW column respectively.

[0012] The immunotoxins expressed and purified in the present invention can be used in a method of inducing immune tolerance. It would be desirable to provide a method of expression and purification that increased the yield of immunotoxins. The present invention addresses this problem and others in the manner described below.

SUMMARY OF THE INVENTION

[0013] In one aspect, the present invention relates to a method of expressing an immunotoxin in *Pichia pastoris* comprising a) growing the *Pichia pastoris* in a growth medium comprising an enzymatic digest of protein and yeast extract; and b) performing methanol induction of the *Pichia pastoris* with a methanol and glycerol containing feed, wherein the *Pichia pastoris* is contacted with a phenylmethanesulfonyl fluoride and a source of amino acids and wherein the methanol induction is at a temperature of below about 17.5°C.

[0014] In another aspect, the present invention relates to a method of expressing an immunotoxin in Pichia pastoris with a mutation in the amino acid sequence encoding EF-2 comprising a) growing the Pichia pastoris with a mutation in the amino acid sequence encoding EF-2 in a growth medium comprising an enzymatic digest of protein and yeast extract; and b) performing methanol induction of the Pichia pastoris with a mutation in the amino acid sequence encoding EF-2 with a methanol containing feed, wherein the Pichia pastoris with a mutation in the amino acid sequence encoding EF-2 is

contacted with a phenylmethanesulfonyl fluoride and a source of amino acids and wherein the methanol induction is at a temperature of below about 17.5°C.

[0015] In yet another aspect, the present invention relates to a method of purifying a non-glycosylated immunotoxin comprising a) loading a solution containing the non-glycosylated immunotoxin onto a hydrophobic interaction column; b) obtaining a first non-glycosylated immunotoxin containing eluant from the hydrophobic interaction column; c) loading the non-glycosylated immunotoxin containing eluant from step (b) onto an anion exchange column; d) obtaining a second non-glycosylated immunotoxin containing eluant from the anion exchange column by eluting the non-glycosylated immunotoxin with a sodium borate solution; e) diluting the concentration of sodium borate in the second non-glycosylated immunotoxin containing eluant from step (d) to about 50 mM or less; f) concentrating the diluted non-glycosylated immunotoxin containing eluant from step (e) over an anion exchange column; and g) obtaining a purified non-glycosylated immunotoxin from the anion exchange column.

[0016] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention.

[0018] Fig. 1. (A) Conservation of diphthamide domain and DT-resistant mutations in eukaryotic EF-2s. (B) Nucleotide sequence mutations for the substitution of Arg for Gly 701 in *Pichia pastoris* EF-2. The underlined sequences are the sites for the restriction enzyme *Sac* II that resulted from the nucleotide mutations.

[0019] Fig. 2. The 5' end sequence of *Pichia pastoris* EF-2 showing the short intron (SEQ ID NO:11) and (SEQ ID NO:12). The 5' splice site, branch site and and 3' splice site are underlined. The EF-2 coding sequence is in bold.

[0020] Fig. 3. (A) (B) (C) (D) Nucleotide and deduced amino acid sequence of *Pichia pastoris* EF-2 (SEQ ID NO:13). The nucleotide sequence is numbered from the beginning of the initiation codon. Consensus GTP-binding motif in the protein sequence is AHVDHGKST (SEQ ID NO:14), the threonine residue putatively phosphorylated *in vivo* by EF-2 kinase is circled and the effector domain conserved among all elongation factors is DEQERGITIKSTA (SEQ ID NO:15). The 22 well-conserved residues of the diphthamide domain are boxed.

[0021] Fig. 4. Targeted mutation using the 3' sequence of EF-2 that has been mutated *in vitro*. The mutating plasmid pBLURA-Δ5'mutEF-2 contains four essential elements: β-lactamase gene(Ampr), Uracil selection marker (URA3), 3'AOX1 transcription termination sequence(TT) and the *in vitro* mutated FF-2 3' sequence, Δ5'mutEF-2.

[0022] Fig. 5. Agarose gel electrophoresis of of PCR products of selected Ura+ clone derived from *Pichia pastoris* JC308 strain. (A) PCR products with primers 1 and m; (B) PCR products with primers 2 and w; (C) Sac II digested PCR products with primers 2 and 3.

[0023] Fig. 6. Western blot analysis of cytosolic expression of DT-A chain in mutated and wild type *Pichia pastoris* strains. (A) Lanes 1-6 are the cell extracts of 6 independent clones of mutEF2JC307-8 transformed with pPIC3-DtA, +C: The purified A-dmDT390-bisFv. M: SeeBlue plus2 Protein markers (Invitrogen). (B) Cytosolic expression of DT-A chain in cultures of two separated colonies of mut-3 and mut-5 that are mutEF2JC307-8(3) and (5) respectively, C3 and C4. Protein samples are loaded on 4-12% NuPAGE gels (Invitrogen).

[0024] Fig. 7. The effect of intra-cellular expression of DT-A on the survival of *Pichia pastoris* strains with mutated or wild type EF-2. Mut-3 and Mut 5 are EF-2 mutants mutEF2JC307-8-DtA(3) and (5) respectively, Mut-3 expressed DT A chain in the cytosol, mut-5 did not. C3 and C4 are the wild type EF-2 strains that did (C4) or did not express DT A chain in the cytosol. The first bar in each category indicates the colony-forming units before methanol induction. The second bar in each category represents the colony-forming units after methanol induction.

[0025] Fig. 8. Schematic presentation of plasmid construction. (A) pBLARG-A-dmDT390-bisFv; (B) pPGAPArg-A-dmDT390-bisFv; (C) pPGAPHis-A-dmDT390-bisFv.

[0026] Fig. 9. Western blot analysis of expression of A-dmDT390-bisFv. Samples of culture media (a) and cell extracts (b) were loaded on 4-12% NuPAGE gels (Invitrogen). Lanes of +c are purified A-dmDT390-bisFv. Lanes 1-9 were samples of 9 selected clones of mut EF2JC303 transformed with 2 copies of the A-dmDT390-bisFv gene. Lanes 10, 11 and 12 were samples of single copy clones: lane 12 was the non-mutated EF-2 clone JHW#2, lanes 10 and 11 were two of selected clones of mutEF2JC307-8(1) and mutEF2JC307-8(2) that is also called YYL#8-2.

[0027] Fig. 10. Comparison of the methanol consumption rate among different *Pichia pastoris* strains. All of these strains are Mut+ (Methanol utilization plus) except for pJHW#3, which is MutS (Methanol utilization slow). pJHW#2 to 5 and the EF-2 mutant YYL#8-2 all expressed the bivalent immunotoxin A-dmDT390-bisFv. X-33 is a wild type strain that does not express A-dmDT390-bisFv, but was transformed with the expression vector.

[0028] Fig. 11. Comparison of profiles of methanol consumption rate between X-33 and JW102 and between different nutrient feeding of JW102 at the indicated temperature. YE and casa represents feeding of yeast extract and casamino acids, respectively.

[0029] Fig. 12. Lowering agitation speed in fermentation reduces immunotoxin aggregates. Fermentation performed at high agitation speed resulted in more than 50% of the secreted immunotoxin being present in inactive aggregate forms in the supernatants. In addition, aggregates accumulated over induction time. However, lowering agitation speed from 800 rpm to 400 rpm reduced immunotoxin aggregates. Immunotoxin aggregates were maintained at the same level over the induction time.

[0030] Fig. 13. Effect of Tween 20 on aggregation of purified immunotoxin after 20 hrs incubation at 30°C at 250 rpm. Using purified immunotoxin, Tween 20 prevented the formation of aggregates by agitation. Approximately 50% of the purified immunotoxin was aggregated by incubation at 30 C at 250 rpm for 20 hours. However,

0.01%~0.04% of Tween 20 significantly reduced the aggregation of purified immunotoxin by agitation.

[0031] Fig. 14. Change of gain of wet cell density during the first 44 hours of methanol induction. MeOH, methanol alone and feeding of casamino acids; M:G=4:1, methanol/glycerol mixed feeding and feeding of casamino acids; YE+MeOH, feeding of yeast extract and methanol alone; YE+4:1, feeding of yeast extract and methanol/glycerol mixed feeding.

[0032] Fig. 15. Expression level of the bivalent immunotoxin and its final purification yield depending on induction temperature. (A) change of expression level by induction temperature. (B) change of the final purification yield from 1 liter of supernatant taken at 22, 44, and 67 hours of methanol induction. 22 hrs, 44 hrs, and 67 hrs represent time of methanol induction. (C) change of methanol consumption depending on induction temperature.

[0033] Fig. 16. A representative of optimized fermentation runs. Samples taken at indicated induction time points were fractionated on 4-20% SDS-tris-glycine gel and the gel was stained with Coomassie blue dye. Arrow indicates the position of the bivalent immunotoxin. Mark 12 marker (Invitrogen) was used.

[0034] Fig. 17. SDS-PAGE analysis of proteins obtained by butyl 650M capture step. Lane 1~4, sample flow-through fraction #1~#4; lane 5, pooled sample flow-through fractions; lane 6~8, wash fraction #1~#3; lane 9, pooled wash fractions; lane 10, 11, 17, supernatant; lane 12, Mark 12 protein standards (Invitrogen); lane 13~15, eluted fraction #1~#3; lane 16, pooled eluted fractions. IT, immunotoxin.

[0035] Fig. 18. SDS-PAGE analysis of proteins obtained by Poros 50 HQ borate anion exchange step. Lane 1, Mark 12 protein standards (Invitrogen); lane 2, sample obtained from Butyl 650M HIC step; lane 3~7, sample flow-through fraction #1~#5; lane 8, fraction #1 eluted with 25 mM borate in Buffer B; lane 9, fraction #2 eluted with 50 mM borate in Buffer B; lane 10, fraction #3 eluted with 75 mM borate in Buffer B; lane 11, fraction #4 eluted with 100 mM borate in Buffer B; lane 12, fraction #5 fraction eluted with 1 M NaCl in Buffer B. IT, immunotoxin.

[0036] Fig. 19. Analytical gel filtration and SDS-PAGE analysis of purified immunotoxin. A: Chromatogram of Superdex 200 10/300 GL gel filtration. B: Picture of Coomassie-stained SDS-polyacrylamide gel.

[0037] Fig. 20. (A) (B) (C) Amino acid sequence of Ala-dmDT390bisFv(UCHT1) (SEQ ID NO:16).

DETAILED DESCRIPTION OF THE INVENTION

[0038] Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods or specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0039] As used in the specification and the appended claims, the singular forms "a," "an" and "the" include the plural forms unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

[0040] Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is

disclosed the "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15.

[0041] In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

[0042] "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

[0043] "Contacted" means one substance is placed in physical association with another substance.

[0044] "Non-glycosylated" means in the absence of glycosylation or in the absence of glycosylation perceptible using routine methods known in the art for measuring glycosylation. Thus non-glycosylated includes having had glycosylation sites mutated so that glycosylation does not occur, expressing in a system in which glycosylation will not occur or not possessing glyscosylation sites in the wild type state.

[0045] "Loading" a column means placing the sample in a position in which at least a portion of the sample will eventually enter the part of the column occupied by the resin.

[0046] The invention provides a system for expressing and purifying mutant ADP ribosylating toxins and toxin fusion proteins in a *Pichia pastoris* mutant. The methods of the present invention possess the advantage of being compliant with Good Manufacturing Practices.

[0047] The invention provides a method of expressing an immunotoxin in *Pichia pastoris* comprising growing the *Pichia pastoris* in a growth medium comprising an enzymatic digest of protein (e.g., soy protein) and yeast extract; and performing methanol

induction of the *Pichia pastoris* with a methanol and glycerol containing feed (e.g., with a methanol to glycerol ration of about 4:1), wherein the *Pichia pastoris* is contacted with a phenylmethanesulfonyl fluoride and a source of amino acids (e.g., a yeast extract) and wherein the methanol induction is at a temperature of below about 17.5°C.

[0048] As used throughout, optionally, the immunotoxin is a fusion protein. The immunotoxin can comprise a diphtheria toxin moiety. The toxin moiety can be a truncated moiety and/or can comprise mutations as compared to the wild-type toxin. The immunotoxin can further comprise a CD3 antibody moiety or other antibody moiety. In a preferred embodiment the immunotoxin comprises A-dmDT390-bisFv(G4S).

[0049] The contacting step of the expression method includes contact with the phenylmethanesulfonyl fluoride and the source of amino acids for at least 2 hours, including 2, 3, 4, 5, 6, 7, 8, 9, 10, or more hours or any amount in between. Preferably, the phenylmethanesulfonyl fluoride is dissolved in the 4:1 methanol glycerol induction feed and the concentration does not exceed 10 mM.

[0050] The methanol induction temperature is preferably below about 17.5, and even more preferably is about 15°C. Other temperatures include 16.5, 16.0, 15.5, 14.5, 14.0, 13.5, 13, 12.5, 12°C or any amounts in between.

[0051] In a preferred embodiment, the composition of the growth medium is about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate without amino acids, and about 0.435% PTM1 solution. Optionally, the growth medium further comprises an antifoaming agent. More specifically, the antifoaming agent is present at a concentration of about 0.01% or greater. Thus, the composition of the growth medium can be about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate without amino acids, about 0.435% PTM1 solution and about 0.01% antifoaming agent.

[0052] Optionally, the dissolved oxygen concentration is maintained at a value of 40% or higher in the expression method of the invention. Furthermore, the growth step is optionally at a pH of about 3.5 and the methanol induction step is at a pH of about 7.0.

[0053] Also provided by the invention is a method of purifying a non-glycosylated immunotoxin comprising (a) loading a solution containing the non-glycosylated immunotoxin onto a hydrophobic interaction column; (b) obtaining a first non-glycosylated immunotoxin containing eluant from the hydrophobic interaction column; (c) loading the non-glycosylated immunotoxin containing eluant from step (b) onto an anion exchange column; (d) obtaining a second non-glycosylated immunotoxin containing eluant from the anion exchange column by eluting the non-glycosylated immunotoxin with a sodium borate solution; (e) diluting the concentration of sodium borate in the second non-glycosylated immunotoxin containing eluant from step (d) to about 50 mM or less; (f) concentrating the diluted non-glycosylated immunotoxin containing eluant from step (e) over an anion exchange column; and (g) obtaining a purified non-glycosylated immunotoxin from the anion exchange column. Optionally, the method further comprises washing the anion exchange column with about 25 mM sodium borate solution prior to eluting with the sodium borate solution. Preferrably the non-glycosylated immunotoxin being purifed is expressed by the methods taught herein.

[0054] The concentration of the sodium borate solution in step (d) of the purification method is between about 50 mM and about 200 mM, and preferably is between about 75 mM and about 100 mM. Even more preferably, the concentration of sodium borate in step (e) is about 20 mM.

[0055] The bivalent anti-T cell immunotoxin, A-dmDT390-bisFv(G_4S), which selectively kills human T cells, was developed for treatment of T-cell leukemia, autoimmune diseases and tolerance induction for transplantation (U.S. Patent Application No. 09/573,797, incorporated by reference). The bivalent anti-T cell immunotoxin, A-dmDT390-bisFv(G_4S), consists of the first 390 amino acid residues (DT390) of diphtheria toxin (DT) and two antigen-binding domains (sFv) of the anti-CD3ε antibody, UCHT1 (Thompson et al., 2001). The first 390 amino acid residues of DT (DT390) contains catalytic domain or A chain of DT that inhibits protein synthesis by ADP-ribosylation of EF-2, and the translocation domain that translocates the catalytic domain to the cytosol by interactions with cytosolic Hsp90 and thioredoxin reductase (Ratts et al., 2003). The two tandem sFv molecules are responsible for binding of immunotoxin to the CD3ε subunit in the complex of TCR on human T cells. The linker (L) can be a Gly-

Ser linker. The Gly-Ser linker can be but is not limited to (Gly4Ser)n or (Gly3Ser)n. More specifically, the linker can be a (Gly4Ser)3 linker (GGGGSGGGGSGGGGS) (SEQ ID NO:17), also referred to herein as (G4S), or a (Gly3Ser)4 linker (GGGSGGGSGGGSGGGS) (SEQ ID NO:18), also referred to herein as (G3S). Two N-glycosylation sites in the immunotoxin have been removed by introduction of two mutations (Liu et al., 2000), resulting in a non-glycoprotein with a molecular weight of 96.5 kDa. The immunotoxin is sensitive to pH levels below 6.0, as shown by the fact that low pH induces an irreversible conformational change in the translocation domain of the DT390 moiety. The translocation domain mediates translocation of the A chain in the DT390 from the endosomes or the plasma membrane to the cytosol in a proton dependent manner. The catalytic A chain is responsible for protein synthesis inhibition by ADP-ribosylation of elongation factor 2 (EF-2) in the cytosol. The pH sensitivity of the immunotoxin restricts the use of cation exchange chromatography and affinity chromatography based on eluting with a low pH buffer.

[0056] The multi-domain structure of the bivalent immunotoxin hinders efficient production in *Escherichia coli*, and most eukaryotes are sensitive to the toxin. *Pichia pastoris* is a good expression system for the bivalent anti-T cell immunotoxin A-dmDT390-bisFv as it provides optimal protein folding compared to prokaryotic expression systems and provides higher yields compared to mammalian cell expression (CHO cells). Antibody fusion proteins require correct disulfide bridges and the endoplasmic reticulum of yeast provides an oxidizing environment like that of eukaryotic antibody producing cells. The multi-domain structure of the bivalent immunotoxin requires a eukaryotic expression system to properly fold this complex protein. Yet most eukaryotes are sensitive to the effects of protein synthesis inhibition upon expression of the immunotoxin. However, a budding yeast, *Pichia pastoris* has a certain degree of tolerance to DT (Neville et al., 1992; Woo et al., 2002; Woo and Neville, 2003) and yielded the immunotoxin at a level of 40 mg/L in fermentor culture. The immunotoxin was produced by fermentation of genetically engineered *Pichia pastoris* (JW102, renamed from pJHW#2 (Woo et al., 2002)) via the secretory route.

[0057] The immunotoxin was produced in *Pichia pastoris* (JW102) via the secretory route under control of the AOX1 promoter in the fermentor using methanol as a

carbon source. There were two major impediments to efficient immunotoxin production, the toxicity of the immunotoxin towards *Pichia pastoris* and the limited secretory capacity of *Pichia pastoris* for this immunotoxin. The toxicity towards *Pichia pastoris* resulted in a decrease in the metabolic rate of methanol consumption, a cell growth rate reduction and very low productivity in a defined medium during methanol induction. These problems were overcome by (1) using an enzymatic digest of soy protein and yeast extract based complex medium, (2) using methanol/glycerol mixed feed (4:1) to supplement energy source during methanol induction, and (3) continuously feeding PMSF and yeast extract during methanol induction. Lowering the induction temperature to 15 °C improved secreted immunotoxin yield by almost 2-fold, up to 40 mg/L, even though methanol consumption was reduced. In addition, the fraction of immunotoxin present as biologically inactive oligomeric forms was decreased. The toxicity of the immunotoxin towards *Pichia pastoris* is attenuated by the use of complex medium. Low temperature methanol induction suppresses overall protein synthesis and this suppression improves secretion efficiency of the immunotoxin in *Pichia pastoris* by balancing immunotoxin input and output through the secretory pathway.

[0058] After gene optimization to reduce the AT content of the DNA sequence, secreted expression levels under the AOX1 promoter of 25- 30 mg/L can be obtained in bioreactors after 24-44 hours of induction. *Pichia pastoris* was sensitive to the toxic effects of cytosolic expressed diphtheria toxin A chain which ADP ribosylates elongation factor 2 (EF-2) leading to cessation of protein synthesis. Noticeable toxicity to expression of A-dmDT390-bisFv by the secretory route was limited to a continuous fall in methanol consumption after induction. A mixed feed of glycerol and methanol was provided to the cells. Expression of the catalytic domain (A chain) of DT in the cytosol is lethal to *Pichia pastoris*. When cells bearing the construct A-dmDT390-bisFv (UCHT1) were induced by methanol to express the immunotoxin, nearly 50% are killed after 24 hours (Woo et al., 2002). In contrast, when the same immunotoxin was expressed in CHO cells that had been mutated to DT resistance, no toxic effect was observed (Liu, et al., 2000; Thompson, et al., 2001). In the cytosol of eukaryotes, the catalytic domain of DT catalyzes ADP ribosylation of elongation factor 2 (EF-2), leading to inhibition of protein synthesis and cell death (by protein starvation and or apoptosis, Van Ness et al., 1980;

Houchins, 2000). The sensitivity of the eukaryotic EF-2 to ADP-ribosylation by these toxins lies in the structure of protein. EF-2 is a single polypeptide chain of about 850 amino acids and is composed of two domains. The N-terminal G domain is responsible for binding and hydrolysis of GTP that promotes translation, and the C-terminal R (or diphthamide) domain is thought to interact with the ribosome (Kohno et al., 1986; Perentesis et al., 1992). The diphthamide domain (Fig.1a) contains a histidine residue in a region of 22 residues that are well conserved in the EF-2 of all eukaryotes. This conserved histidine is specifically modified post-translationally to the derivative, diphthamide, which is the unique target for ADP-ribosylation by DT (Van Ness et al., 1880). In *S. cerevisiae*, the conserved histidine can be mutated and substitutions with some other 2 amino acids yielded functional EF-2s that were resistant to ADP-ribosylation (Phan et al., 1993; Kimata and Kohno 1994). However, cells with EF-2 mutated at diphthamide grew more slowly than those expressing wild-type EF-2. In CHO cells, a single substitution of arginine for glycine, which is another well conserved residue located at the 3rd position to the C-terminal side of the diphthamide, also prevented the formation of diphthamide (Kohno & Uchida, 1987; Foley et al., 1992) and resulted in non-ADP-ribosylatable EF-2. This mutation had the same effect on EF-2 of *S. cerevisiae* (Kimata et al., 1993). In contrast to the mutation at diphthamide, the Gly to Arg mutation in EF-2 did not affect cell growth of CHO and *S. cerevisiae* (Foley et al., 1992; Kimata and Kohno 1994; Kimata et al., 1993).

[0059] In order to determine if the expression level of A-dmDT390-bisFv could be further increased by rendering *Pichia pastoris* insensitive to toxin, the EF-2 gene of *Pichia pastoris* has been mutated so that the Gly at position 701 was changed to Arg, which has been shown to prevent ADP-ribosylation of EF-2 in other organisms. The EF-2 mutagenesis required cloning of the gene, introduction of the *in vitro* mutated sequence with a selection marker, URA3, to the genome and PCR identification of mutated clones. The entire EF-2 gene of *Pichia pastoris* has been cloned and sequenced. The coding sequence of *Pichia pastoris* EF-2 is 2526 nucleotides coding for 842 amino acids. The *Pichia pastoris* EF-2 is the same as the EF-2 of *S. cerevisiae* and *S. pombe* in length and shares 88% and 78% of identity in amino acid sequence with these two, respectively. In contrast to these two yeasts, *Pichia pastoris* has only one copy of the EF-2 gene that

contains a short intron. Before the complete sequence of EF-2 was known, different approaches were used to mutate *Pichia pastoris* to obtain DT resistant strains. All these effort were unsuccessful due to the lack of robust selection. Based on the EF-2 sequence obtained, a pBLURA-Δ5'mutEF-2 was constructed that targets *Pichia pastoris* EF-2 gene and introduces a mutation of Gly 701 to Arg to the gene by homologous recombination. The construct contains the 3' end 1028 nucleotides of EF-2 that has been mutagenized *in vitro* to contain the amino acid substitution and the auxotrophic marker URA3. A PCR detection method was also developed for fast and accurate identification of mutant clones after uracil selection. The targeted mutation strategy with construct pBLURA-Δ5'mutEF-2 allowed mutation of the EF-2 gene of *Pichia pastoris* with about 40% of uracil positive clones being found to contain the introduced mutations. EF-2 mutants were developed with different auxotrophic markers, (specifically mutEF2JC308 (adel arg4 his4), mutEF2JC303 (arg4 his4) and mutEF2JC307 (his4)) and demonstrated that the Gly 701 to Arg mutation in EF-2 confers resistance to the cytosolic expression of DT A chain. When EF-2 mutants were used to express A-dmDT390-bisFv under the control of AOX1 promoter, they did not show the advantage over the non-mutated expressing strain JW102 in the production of the protein in shake-flask. However, in large-scale fermentation culture under conditions adopted from those optimal for JW102, the production of the mutant strain YYL#8-2 [mutEF2JC307-8(2)] increased continuously for 96 hours and reached a level 1.46-fold greater than the non-mutated JW102 strain. Cell growth and methanol consumption rates of the mutant strain expressing A-dmDT390-bisFv were the same as that of the non-expressing wild type strain. Therefore it appears that expression of A-dmDT390-bisFv is not toxic to the mutant strain. Production of A-dmDT390-bisFv in the mutant strain can be increased by optimizing the fermentation conditions, such as the time to start methanol induction and the feeding of medium components and protein inhibitors. The EF-2 mutants allowed expression of A-dmDT390-bisFv under the control of the constitutive GAP promoter (P_{GAP}). In shake-flask culture, the production of A-dmDT390-bisFv under P_{GAP} was about 30% higher than that under P_{AOX1}. The increase in production under P_{GAP} may be more significant in fermentation cultures since fermentation allows cells to grow to very high density.

[0060] In the *Pichia pastoris* expression system, most heterologous proteins such as botulinum neurotoxin fragments for vaccine use (Potter et al., 2000), hepatitis B surface antigen (Hardy et al., 2000), gelatin (Werten et al., 1999), collagen (Nokelainen et al., 2001), and insulin (Wang et al., 2001) were successfully expressed and/or secreted by using a simple defined medium. The cytosolic expression of the catalytic domain of DT causes protein synthesis inhibition, leading to complete cell death in the defined medium, but not in complex media (Liu et al., 2003). This finding indicates that complex media play a role in attenuation of protein synthesis inhibition that is caused by ADP-ribosylation of EF-2. A very low production of the bivalent immunotoxin was observed in the defined medium but not in a complex medium in shake flask culture. Fermentation of *Pichia pastoris* for expression of heterologous proteins had been developed on the basis of a defined medium but use of complex media for expression of the bivalent immunotoxin in a secreted form provides a higher level of production.

[0061] In the large scale production of bivalent immunotoxin in *Pichia pastoris*, lowering the induction temperature to 15 °C substantially improves the secretion of bioactive immunotoxin, and thereby partially compensates for the limitation in *Pichia pastoris* secretory capacity. In addition, the use of complex medium containing yeast extract further enhances immunotoxin secretion, apparently by attenuating the toxic effects of the immunotoxin on the *Pichia pastoris* host.

[0062] The expression level of the bivalent immunotoxin was improved by 4-fold in bioreactor culture compared to shake flask culture by optimizing the fermentation conditions in *Pichia pastoris* as follows: (1) use of Soytone Peptone and yeast extract based complex medium, (2) use of methanol/glycerol mixed feed (4:1) to supplement the energy source during methanol induction, (3) continuous feeding of PMSF and yeast extract during induction, and (4) lowering temperature to 15 °C during methanol induction.

[0063] A major problem in production of the bivalent immunotoxin was reduction of methanol utilization during the methanol induction phase. The reduction of methanol utilization results from a reduction in the activity of the rate limiting enzyme, alcohol oxidase (AOX1). This could be secondary to protein synthesis inhibition by the bivalent

immunotoxin reaching the cytosol compartment through leakage from the secretory compartment or by proton dependent translocation from the mildly acidic secretory compartment (Arata et al., 2002). The fact that methanol utilization is not affected by immunotoxin production in a *Pichia pastoris* strain mutated to toxin resistance in the EF-2 gene (Liu et al., 2003) indicates that toxin induced ADP-ribosylation is the cause of the decreased AOX1 activity in strain JW102. However, control of AOX1 level is balanced by both synthesis as well as degradation, and degradative mechanisms could be augmented in response to toxin mediated ADP-ribosylation. For reasons unknown, yeast extract increased methanol utilization, though not to wild type levels. In addition, low methanol utilization negatively affected *Pichia pastoris* cell growth. This was corrected by adding another carbon source, glycerol, and continuous feeding of yeast extract during methanol induction. These two corrections raised the wet cell density to 80 % of the non-expressing strain.

[0064] As described above, in order to compensate for *Pichia pastoris* protein synthesis inhibition by the expressed immunotoxin, the initial focus was on maintenance of constant methanol consumption during methanol induction. The fermentation conditions were manipulated for full activation of alcohol oxidase I (AOX1), the rate limiting enzyme for methanol metabolism (Veenhuis et al., 1983). Since the immunotoxin gene was under the control of the same strong promoter as the AOX1 gene, the immunotoxin should be highly expressed. However, it has previously been observed in the secretion expression of heterologous proteins that each protein appears to have an optimal secretion level. Expression beyond the optimal level (overexpression) of secreted heterologous proteins can cause a reduction in secreted protein yields in mammalian, insects and yeast cells (Bannister and Wittrup, 2000; Liebman et al., 1999; Liu et al., 2003; Pendse et al., 1992). In order to determine whether the bivalent immunotoxin was being overexpressed in *Pichia pastoris*, the inventor lowered the induction temperature during methanol induction. Since most cellular activities including protein synthesis are decreased at low temperature, lowering induction temperature should decrease the synthetic rate of the bivalent immunotoxin. Any resulting change in secretion rate was judged. Bivalent immunotoxin secretion was increased at low induction temperatures, reaching a maximum at 17.5 °C and secretion of bioactive

immunotoxin reached a maximum at 15 °C, in spite of the fact that methanol consumption rate fell to 75% of its 23 °C value. Because continuous feeding of PMSF and yeast extract during induction effectively inhibited protease activity in supernatants, it appears unlikely that a reduction in protease activity with lower induction temperature accounts for the nearly 2-fold increase in bivalent immunotoxin secretion seen at 15 °C. The limitation in *Pichia pastoris* secretion of bivalent immunotoxin previously described may actually represent an overexpression at 23 °C that is reduced at 15 °C achieving a better balance of input and output within the secretory compartment.

[0065] A major problem encountered in the large scale purification of the bivalent anti-T cell immunotoxin, A-dmDT390-bisFv(G_4S), from *Pichia pastoris* supernatants was the presence of host glycoproteins exhibiting similar charge, size and hydrophobicity characteristics. This problem was overcome by employing borate anion exchange chromatography. Borate anion has an affinity for carbohydrates and imparts negative charges to these structures. At a concentration of sodium borate between 50 and 100 mM, the non-glycosylated immunotoxin did not bind to Poros 50 HQ anion exchanger resin, but glycoproteins, including aggregates related to the immunotoxin, did bind. By using this property of the immunotoxin in the presence of sodium borate, a 3-step purification procedure was developed: (1) Butyl 650M hydrophobic interaction chromatography, (2) Poros 50 HQ anion exchange chromatography in the presence of borate, and (3) Q anion exchange chromatography. The final preparation exhibited a purity of greater than 98% and a yield of over 50% from the supernatant. Previously, boronic acid resins have been used to separate glycoproteins from proteins. However, combining borate anion with conventional anion exchange resins accomplishes separation of the immunotoxin from glycoproteins, and eliminates the need to evaluate non-standard resins with respect to good manufacturing practice guidelines.

[0066] The immunotoxin is functionally active only in its monomeric form. However the supernatant harvested from the fermentation run contained monomeric, dimeric and higher oligomeric forms of the immunotoxin as well as *Pichia pastoris* proteins. Among these *Pichia pastoris* proteins, a glycoprotein species of approximately 45 kDa was present in dimeric form (~90 kDa). The dimeric and higher oligomeric forms of the immunotoxin were relatively easy to separate by the use of conventional

hydrophobic interaction chromatography and anion exchange chromatography. However, it was difficult to isolate the pure immunotoxin because the 45 kDa glycoproteins were very similar to the immunotoxin in size, hydrophobicity, and isoelectric point.

[0067] Previously, immobilized phenylboronate resins have been used for separation of glycoproteins from proteins (Myohanen et al., 1981; Bouritis et al., 1981; Williams et al., 1981; Zanette et al., 2003). These immobilized resins bind and selectively retard glycoproteins depending on pH, presence of sugar, type of sugar, concentration of sugar and buffer species. Borate anion exchange chromatography is used rather than the immobilized phenylboronate affinity chromatography for separation of the immunotoxin from the 45 kDa glycoprotiens, because of poor separation capability of phenylboronate resin. Borate forms complexes with sugar residues having vicinal cis-hydroxyl groups (Boeseken, 1949) and these complexes are reversible (Weigel, 1963). Reversible complex formation of borate with carbohydrate on glycoproteins resulted in an increased negative charge of the glycoproteins. This property allowed separation of non-glycoproteins and glycoproteins on anion exchange chromatography (Nomoto et al., 1982; Nomoto and Inoue, 1983).

[0068] In large scale purification, conventional anion exchange chromatography by step-eluting with a series of sodium borate buffers enhanced separation of the non-glycosylated immunotoxin from *Pichia pastoris* glycoproteins. The invention includes a scaleable high-yield 3-step purification procedure for the bivalent immunotoxin that incorporates the borate anion exchange step. The procedure contained Butyl 650M hydrophobic interaction chromatography, Poros 50 HQ borate anion exchange chromatography, and Q anion exchange chromatography. This procedure has several advantages: (1) it is a relatively simple process without any dialysis or diafiltration step; (2) it exhibits good repeatability; (3) the final yield is over 50%; and (4) the final purity is over 98%. Development of this procedure was accomplished by the introduction of borate anion exchange chromatography. Borate anion exchange chromatography was used for separation of the immunotoxin from *Pichia pastoris* glycoproteins.

[0069] In a previous study utilizing shake flask culture, the inventor employed a 2-step procedure which involved DEAE anion exchange chromatography and Protein L affinity chromatography for purification of the immunotoxin (Woo et al., 2002). However, the supernatants of high density fermentor cultures of *Pichia pastoris* contain materials that severely reduce the capacity of anion exchange resins. In addition, the Protein L affinity step required excessive column size, was expensive and was not available under Good Manufacturing Practices (GMP) certification. Consequently, alternative procedures were developed. Hydrophobic interaction chromatography using Butyl 650M worked well as a capture step but also concentrated *P. pastoris* glycoproteins having similar charge, size and hydrophobicity as the immunotoxin. Concanavalin A affinity resin was promising for glycoprotein removal, but bleeding of potentially toxic concanavalin A from the resin resulted in unacceptable contamination of the final product.

[0070] It is known that borate reversibly associates to hydroxyl groups of many compounds including carbohydrates, vitamins, coenzymes and ribonucleic acids by formation of complexes that increase the negative charge of the parent compounds (Otsuka et al., 2003; Weith et al., 2003). Based on this property of borate, many applications have been developed. For example, borate was used in capillary electrophoresis for analysis of the oligosaccharide composition of heparin (Desai et al., 1993), for separation of ovalbumin glycoforms (Che et al., 1999), for high voltage paper electrophoresis separation of glycopeptides from ovalbumin (Narasimhan et al., 1980), for immobilized isolectin affinity chromatography for the isolation of trypsin inhibitor from *Echinodorus paniculatus* seeds (Paiva et al., 2003) and for gel filtration analysis of IgG glycopeptides (Rothman and Warren, 1988). Borate anion exchange chromatography was also developed for separation of glycopeptides by using a linear 0~0.4 M sodium borate gradient (Nomoto et al., 1982; Nomoto and Inoue, 1983) and for separation of reduced N-acetylhexosamines by using step elution (Scocca, 1986).

[0071] By using the Poros 50 HQ borate exchange chromatography in the present invention, substantial purification of the monomeric form of the immunotoxin was obtained, even though the immunotoxin in the eluted faction was diluted and a subsequent concentration step by Q anion exchange chromatography was necessary.

[0072] In the separation of the immunotoxin from glycoproteins, borate anion exchange chromatography had different binding characteristics from phenylboronate affinity chromatography. In phenyloboronate affinity chromatography, glycoproteins as well as the immunotoxin were bound under the condition of low ionic strength and they were co-eluted by either 0-100 mM sodium borate gradient or 0-50 mM sorbitol gradient, suggesting that the immunotoxin physically interacts with at least one of the bound glycoproteins, or interacts with the phenylboronate column through an alternate mechanism. The fact that purified bivalent immunotoxin also bound to the phenylboronate column indicates binding through an alternate mechanism.

[0073] Borate anion exchange chromatography is useful for the purification of other *Pichia pastoris* expressed proteins. *Pichia pastoris* is being increasingly used as an expression system for therapeutic recombinant proteins (Cereghino et al., 2002). Many of these recombinant proteins have their glycosylation sites removed due to the profound differences in glycosylation patterns between *Pichia pastoris* and humans. These recombinant proteins are then amenable to purification using borate anion exchange chromatography.

[0074] An immunotoxin used in the present invention comprises a mutant toxin moiety (e.g., DT toxin) linked to an antibody moiety (targeting moiety). Toxins that may be used include but are not limited to diphtheria toxin, ricin toxin, and pseudomonas exotoxin. The antibody moiety is preferably a single chain (sc) variable region.

[0075] Growth medium refers to any substance required for growth of the selected organism. Substances required for growth may include but are not limited to carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur, potassium, magnesium, calcium, sodium, iron, trace elements and organic growth factors. Various materials may be included in growth medium to provide the required substances. Such substances include but are not limited to simple sugars, extracts such as peptone, soytone, tryptone, yeast extract, carbon dioxide, vitamins, amino acids, purines and pyrimidines. A preferred embodiment of the invention utilizes the presence of an enzymatice digest of soy produced by DIFCO.

[0076] An enzymatic digest refers to hydrolysis of a protein or peptide at peptide bonds by one or more of various enzymes. Such enzymes may include but are not limited to trypsin, chymotrypsin, pepsin, thrombin, papain, bromelain, thermolysin, subtilisin or carboxypeptidase A.

[0077] Yeast extract is a preparation of peptides and amino acids obtained by proteolysis of the proteins within yeast cells. A preferred embodiment of the invention utilizes yeast extract produced by DIFCO.

[0078] Induction refers to providing a signal to a given promoter to cause expression of a given gene.

[0079] Feed refers to providing fresh media or nutrients at a rate that at least partially replaces the media or nutrients as they are depleted.

[0080] Moiety refers to one portion of a molecule or compound that is divided into multiple portions. In the present invention, moiety may refer a toxin portion or an antibody portion of an immunotoxin.

[0081] The immunotoxin of the present invention may be expressed in and purified from various organisms. These organisms include yeast such as *Pichia pastoris* or *Sacchromyces cerevisiae*, bacteria such as *Escherichia coli*, mammalian cells such as Chinese hamster ovary cells or baculovirus infected insect cells.

[0082] The hydrophobic interaction column may be but is not limited to a Phenyl–Sepharose® CL-4B, Octyl Agarose, Phenyl–Sepharose 6 Fast Flow, Phenyl-Agarose, Phenyl–Sepharose 6 Fast Flow, Octyl-Sepharose 4 Fast Flow, Butyl Sepharose™ 4 Fast Flow, Octyl Agarose, Phenyl-Agarose, Hydrophobic chromatography media - monoamino MAA-8, Hexyl-Agarose, Dodecyl-Agarose, Hexyl-Agarose, 4-Phenylbutylamine–Agarose, Ethyl–Agarose, Matrix, Butyl–Agarose, Propyl Agarose, Affinity chromatography media AAF-8, Octyl Agarose, Butyl–Agarose, Decyl-Agarose, Phenyl-Agarose, Methyl Matrix: Ceramic HyperD F Hydrogel Composite, Octyl Agarose, Trityl-Agarose, Q Sepharose, Ether 650, Phenyl 650, Butyl 650 or Hexyl 650.. The preferred hydrophobic interaction column is a Butyl 650M hydrophobic interaction column.

[0083] The anion exchange column may be but is not limited to an anion acrylic, anion agarose, anion cellulose, anion dextran or anion polystyrene. The preferred anion exchange columns are a Poros HQ 50 and a Q anion exchange column.

[0084] It is contemplated that any buffer, flow rate, and column size may be used that would successfully effect elution of the immunotoxin in a more pure state than the immunotoxin was loaded upon the column.

[0085] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, protein chemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, including Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL 2nd ed. (Cold Spring Harbor Laboratory Press, 1989); DNA CLONING, Vol. I and II, D. N Glover ed. (IRL Press, 1985); OLIGONUCLEOTIDE SYNTHESIS, M. J. Gait ed. (IRL Press, 1984); NUCLEIC ACID HYBRIDIZATION, B. D. Hames & S. J. Higgins eds. (IRL Press, 1984); TRANSCRIPTION AND TRANSLATION, B. D. Hames & S. J. Higgins eds., (IRL Press, 1984); ANIMAL CELL CULTURE, R. I. Freshney ed. (IRL Press, 1986); IMMOBILIZED CELLS AND ENZYMES, K. Mosbach (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING, Wiley (1984); the series, METHODS IN ENZYMOLOGY, Academic Press, Inc.; GENE TRANSFER VECTORS FOR MAMMALIAN CELLS, J. H. Miller and M. P. Calos eds. (Cold Spring Harbor Laboratory, 1987); METHODS IN ENZYMOLOGY, Vol. 154 and 155, Wu and Grossman, eds., and Wu, ed., respectively (Academic Press, 1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY, R. J. Mayer and J. H. Walker, eds. (Academic Press London, Harcourt Brace U.S., 1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, 2nd ed. (Springer-Verlag, N.Y. (1987), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, Vol. I-IV, D. M. Weir et al., (Blackwell Scientific Publications, 1986); Kitts et al., Biotechniques 14:810-817 (1993); Munemitsu et al., Mol. and Cell. Biol. 10:5977-5982 (1990).

[0086] The present invention utilizes a nucleic acid encoding a diphtheria toxin-containing fusion protein, wherein the nucleic acid can be expressed by a yeast cell.

More specifically, the nucleic acid can be expressed by *Pichia pastoris* or *Sacchromyces cerevisiae*. The nucleic acid capable of being expressed by yeast, comprises a modified native diphtheria-encoding sequence. To promote expression of the nucleic acids of the present invention by yeast cells, regions of the nucleic acid rich in A and T nucleotides are modified to permit expression of the encoded immunotoxin fusion protein by yeast. For example, such modifications permit expression by *Pichia pastoris*. The modifications are designed to inhibit polyadenylation signals and/or to decrease early termination of RNA transcription. More specifically, one or more AT rich regions of the native diphtheria-encoding sequence are modified to reduce the AT content. The AT rich regions include regions of at least 150 contiguous nucleotides having an AT content of at least 60% or regions of at least 90 contiguous nucleotides having an AT content of at least 65%, and the AT content of the AT rich regions is preferably reduced to 55% or lower. The AT rich regions also include regions of at least 150 contiguous nucleotides having an AT content of at least 63% or regions of at least 90 contiguous nucleotides having an AT content of at least 68%, and the AT content of the AT rich regions is reduced to 55% or lower. The native diphtheria-encoding sequence preferably is further modified to encode a diphtheria toxin truncated at its C-terminal. Furthermore, the native diphtheria-encoding sequence preferably is further modified to encode one or more amino acids prior to the amino terminal glycine residue of the native diphtheria toxin. Furthermore, the native diphtheria-encoding sequence preferably is further modified to encode the alph mating factor signal peptide or a protion thereof.

[0087] There are several advantages to yeast expression systems, which include, for example, *Saccharomyces cerevisiae* and *Pichia pastoris*. First, evidence exists that proteins produced in a yeast secretion systems exhibit correct disulfide pairing. Second, efficient large scale production can be carried out using yeast expression systems. The *Saccharomyces cerevisiae* pre-pro-alpha mating factor leader region can be used to direct protein secretion from yeast (Brake, et al.(82)). The leader region of pre-pro-alpha mating factor contains a signal peptide and a pro-segment which includes a recognition sequence for a yeast protease encoded by the KEX2 gene: this enzyme cleaves the precursor protein on the carboxyl side of a Lys-Arg dipeptide cleavage signal sequence. The nucleic acid coding sequence can be fused in-frame to the pre-pro-alpha mating

factor leader region. This construct can be put under the control of a strong transcription promoter, such as the alcohol dehydrogenase I promoter, alcohol oxidase I promoter, a glycolytic promoter, or a promoter for the galactose utilization pathway. The nucleic acid coding sequence is followed by a translation termination codon which is followed by transcription termination signals. Alternatively, the nucleic acid coding sequences can be fused to a second protein coding sequence, such as Sj26 or beta-galactosidase, used to facilitate purification of the fusion protein by affinity chromatography. The insertion of protease cleavage sites to separate the components of the fusion protein is applicable to constructs used for expression in yeast.

[0088] Diphtheria toxin is toxic to yeast when the toxin A chain is synthesized within the cytosol compartment without a secretory signal (Parentesis et al., 1988). This toxin-catalyzed activity is specific for EF-2 and occurs at a unique post-translational histidine residue at the position 699, found in a conserved amino acid sequence in the EF-2 of all eukaryotes. A change of glycine to arginine residue at the position 701 in yeast EF-2 results in resistance to DT.

[0089] (Ala)dmDT390-bisFv(UCHT1) was produced in the *Pichia* medium at a level of 5mg/ml whether or not the mutant EF-2 gene was present. There is an extremely tight coupling between the presence of the alpha-mating factor signal sequence and the compartmentalization of (Ala)dmDT390 -bisFv(UCHT1) into the secretory pathway and away from the EF-2 toxin substrate in the cytosol compartment, since one molecule of toxin in the cytosol can inactivate 99% of the EF-2 in 24 hours. Producing (Ala)dmDT390-bisFv(UCHT1) in *Pichia* utilizing the alpha-mating factor signal sequence without mutating the *Pichia* to toxin resistance provided a successful outcome. Another combination of a yeast produced toxin (ricin A chain) and signal sequence, Kar2, resulted in death of the producing cells (Simpson et al., 1999 (80)). It is possible that, at higher gene dosages of immunotoxin fusion protein in *Pichia*, mEF-2 may confer a benefit in production. A further advantage of yeast over mammalian cells for immunotoxin fusion protein production is the fact that intact yeast are highly resistant to diphtheria toxin present in the external medium to levels as high as 3.3 x 10-6 M. Evidently the yeast capsule prevents retrograde transport of toxin back into the cytosol compartment as occurs in mammalian cells and in yeast spheroplasts (Chen et al. 1985).

[0090] The invention may utilize a cell comprising a nucleic acid that encodes the immunotoxin fusion protein. The cell can be a prokaryotic cell, including, for example, a bacterial cell. More particularly, the bacterial cell can be an *E. coli* cell. Alternatively, the cell can be a eukaryotic cell, including, for example, a Chinese hamster ovary (CHO) cell (including for example, the DT resistance CHO-K1 RE 1.22c cell line, as selected by Moehring & Moehring), myeloma cell, a *Pichia* cell, or an insect cell. The immunotoxin fusion protein coding sequence can be introduced into a Chinese hamster ovary (CHO) cell line, for example, using a methotrexate resistance-encoding vector, or other cell lines using suitable selection markers. Presence of the vector DNA in transformed cells can be confirmed by Southern blot analysis. Production of RNA corresponding to the insert coding sequence can be confirmed by Northern blot analysis. A number of other suitable host cell lines have been developed and include myeloma cell lines, fibroblast cell lines, and a variety of tumor cell lines such as melanoma cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, etc. The vectors containing the nucleic acid segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transformation is commonly utilized for prokaryotic cells, whereas calcium phosphate, DEAE dextran, or lipofectin mediated transfection or electroporation may be used for other cellular hosts.

[0091] The nucleic acids used in the present invention can be operatively linked to one or more of the functional elements that direct and regulate transcription of the inserted nucleic acid and the nucleic acid can be expressed. For example, a nucleic acid can be operatively linked to a bacterial or phage promoter and used to direct the transcription of the nucleic acid *in vitro*.

[0092] A mutant strain of *Pichia pastoris* is provided. The mutant strain comprises a mutation in at least one gene encoding elongation factor 2 (EF2). This mutation comprises a Gly to Arg replacement at a position two residues to the carboxyl

side of the modified histidine residue diphthamide. In this manner, the strain is made resistant to the toxic ADP-ribosyating activity of diphtheria toxin.

[0093] A method of expressing a diphtheria toxin protein moiety is provided. Such a method of the invention comprises transfecting a mutated *Pichia pastoris* cell of the invention with a vector comprising a toxin protein-encoding nucleic acid under conditions that permit expressison of the protein-encoding nucleic acid in the cell. The conditions are those used for *Pichia pastoris* cells and can be optimized for the particular system.

[0094] The antibody moiety preferably routes by the anti-CD3 pathway or other T cell epitope pathway. The immunotoxin can be monovalent, but divalent antibody moieties are presently preferred since they have been found to enhance cell killing by about 15 fold. It is contemplated that any number of chemical coupling or recombinant DNA methods can be used to generate an immunotoxin of the invention. Thus, reference to a fusion toxin or a coupled toxin is not necessarily limiting. The immunotoxin can be a fusion protein produced recombinantly. The immunotoxin can be made by chemical thioether linkage at unique sites of a recombinantly produced divalent antibody (targeting moiety) and a recombinantly produced mutant toxin moiety. The targeting moiety of the immunotoxin can comprise the human μCH2, μCH3 and μCH4 regions and VL and VH regions from murine Ig antibodies. These regions can be from the antibody UCHT1 so that the antibody moiety is scUCHT1, which is a single chain CD3 antibody having human μCH2, μCH3 and μCH4 regions and mouse variable regions as shown in the figures. Numerous DT mutant toxin moieties are contemplated, including for example, DT390 and DT389, with a variety of mutations or as the wild type toxin moiety.

[0095] The toxin moiety retains its toxic function, and membrane translocation function to the cytosol in full amounts. The loss in binding function located in the receptor binding domain of the protein diminishes systemic toxicity by reducing binding to non-target cells. Thus, the immunotoxin can be safely administered. The routing function normally supplied by the toxin binding function is supplied by the targeting antibody anti-CD3. The essential routing pathway is (1) localization to coated pits for endocytosis, (2) escape from lysosomal routing, and (3) return to the plasma membrane.

[0096] Any antibody that can route in this manner will be effective with the toxin moiety, irrespective of the epitope to which the antibody is directed, provided that the toxin achieves adequate proteolytic processing along this route. Adequate processing can be determined by the level of cell killing.

[0097] When antibodies dissociate from their receptors due to changes in receptor configuration induced in certain receptors as a consequence of endosomal acidification, they enter the lysosomal pathway. This can be prevented or minimized by directing the antibody towards an ecto-domain epitope on the same receptor which is closer to the plasma membranes (Ruud, et al. (1989) Scand. J. Immunol. 29:299; Herz et al. (1990) J. Biol. Chem. 265:21355).

[0098] The mutant DT toxin moiety can be a truncated mutant, such as DT390, DT389, DT383, DT370 or other truncated mutants, with and without point mutations or substitutions, as well as a full length toxin with point mutations, such as DTM1, or CRM9 (cloned in *C. ulcerans*), scUCHT1 fusion proteins with DTM1 and DT483, DT390, DT389, and DT370 have been cloned and expressed in *E. coli*. The antibody moiety can be scUCHT1 or other anti-CD3 or anti-T cell antibody having the routing and other characteristics described in detail herein. Thus, one example of an immunotoxin for use in the present methods comprises the fusion protein immunotoxin UCHT1 (or a fragment thereof)-DT390.

[0099] There is a consensus sequence for glycosylation (NXS/T (SEQ ID NO:19)) that may be removed or inserted to control glycosylation. Glycosylation occurs in all eukaryotes, e.g. *Pichia pastoris*.

[00100] There are numerous variants of the immunotoxins. Protein variants and derivatives are well understood to those of skill in the art and can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in

the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking *in vitro* or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following are referred to as conservative substitutions.

Amino Acid Abbreviations

Amino Acid	Abbreviations
Alanine	Ala, A
Allosoleucine	AIle
Arginine	Arg, R
Asparagine	Asn, N
aspartic acid	Asp, D
Cysteine	Cys, C
glutamic acid	Glu, E
Glutamine	Gln, K

Glycine	Gly, G
Histidine	His, H
Isolelucine	Ile, I
Leucine	Leu, L
Lysine	Lys, K
Phenylalanine	Phe, F
Proline	Pro, P
Pyroglutamic acid	PGlu
Serine	Ser, S
Threonine	Thr,T
Tyrosine	Tyr,Y
Tryptophan	Trp, W
Valine	Val, V

Amino Acid Substitutions

Original Residue Exemplary Conservative Substitutions, others are known in the art.

Ala	ser
Arg	lys, gln
Asn	gln, his
Asp	glu
Cys	ser
Gln	asn, lys
Glu	asp
Gly	pro
His	asn, gln
Ile	leu, val
Leu	ile, val
Lys	arg, gln;
Met	leu, ile
Phe	met, leu, tyr
Ser	thr

Thr	ser
Trp	tyr
Tyr	trp, phe
Val	ile, leu

[00101] Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in the amino acid substitution table, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

[00102] For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

[0100] Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of

potential proteolysis sites, e.g. Arg, are accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

[0101] Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

[0102] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contains a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In certain transcription units, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

Examples

[0103] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds,

compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Example 1. Transformation with a mutagenizing oligonucleotide.

[0104] The oligomer of 56 nucleotides (see List of Primers) contains two point mutations to change amino acid 701 from glycine to arginine. A mutagenizing oligo (56mer, 100ug) was co-transformed into the GS200 (Mut+, His-, Arg-) strain with an ARG4 DNA fragment. The ARG4 gene with promoter was taken from plasmid PMY30 (supplied by Prof. Jim Cregg, Keck Graduate Institute of Applied Life Sciences, Claremont, CA 91711) by Sph I and EcoR V. Approximately 1000 transformants were obtained. To screen for mutated clones having the correct mutations, diagnostic PCR with primers mdb1EF- 2 and 2253EF-2C was employed. The mutation-detecting primer (mdb1EF-2) can distinguish a difference in DNA sequence between the normal gene and the mutated gene at amino acid 701. For the normal gene, a PCR product could not be produced because 2 nucleotides at the 3' end were not matched with the DNA sequence of the normal gene, preventing extension by the Taq polymerase. For the mutated gene, the primer could anneal perfectly, so Taq polymerase could produce a PCR product. More than 1000 colonies were screened by this PCR method but no mutated colony was identified. (In the above PCR assay, amino acid 701 mutated EF-2 from *S. cerevisiae* served as a positive control. This mutated gene had been made previously with the intent of introducing it into *Pichia pastoris*. However, *Pichia pastoris* thus transformed had a very slow growth rate and produced the protein of interest at low levels.)

[0105] Transformation was performed with a partial DNA fragment containing the conserved region of the EF-2 gene and a mutation on amino acid 701. The partial sequence of *Pichia pastoris* EF-2 (positions 1717 to 2289, Fig. 3 (a) (b) (c) (d)) was mutated *in vitro* to change the amino acid 701 from glycine to arginine (Fig. 1b) and

then co-transformed into the GS200 strain with the ARG4 gene fragment. The inventor obtained more than 2000 Arg4 positive transformants and screened them for the EF-2 mutation by diagnostic PCR with primers mdb2EF-2 and 2253EF-2C. The mutation was not observed.

List of Primers

Primers derived from S. cerevisiae EF-2:

5' primer : TTG GTT ATT GAC CAA ACT AAG GCT GTCCAA (SEQ ID NO:20)

3' primer : ACC TCT CTT CTT GTT TAA GAC GGA GTA GAT (SEQ ID NO: 21)

Primers used in cloning and mutation of *Pichia pastoris* EF-2

dT_{22}-Not: 5'-CTT GCT TTT GCG GCC GCT TTT TTT TTT TTT TTT TTT TTT (SEQ ID NO:22)

EF-2C_2: 5'-G ATA AGA ATG CGG CCG CCA TTT CTT GGT CTT TGG GTT GAA G (SEQ ID NO:23)

EF-2C_1: 5'-GAT AAG AAT GCG GCC GCC AAC TTA GTT GTT GAC CAG TCT AAG (SEQ ID NO: 24)

5'EF-2: 5'-ATA GCT AGC ACT TTG AAG TTC TTA ATT TTG TTC CTC (SEQ ID NO:25)

3'EF-2C: 5'-ATA AGA ATG CGG CCG CAA GTT AAT GAA ACA TTA AGC TTA CAA C (SEQ ID NO: 26)

wEF-2: 5'-G AAT GAC TTG TCC TCC ACC (SEQ ID NO:27)

mEF-2: 5'- G AAT GAC TTG TCC TCC GCG G (SEQ ID NO:28)

EF-1426: 5'-CAA CTA GCT AGC GCT CAC AAC ATG AAG GTC ATG AAA TTC (SEQ ID NO:29)

EF-1318: 5'-AGA ACC GTC GAG CCT ATT GAC GAT (SEQ ID NO:30)

Mutagenizing oligo:

5'-CC CTG CAC GCC GAT GCT ATC CAC AGA AGA GGA GGA CAA GTC ATT CCA ACC ATG AAG (SEQ ID NO:31)

mdb1EF-2: 5'-GCC GAT GCT ATC CAC AGA AGA (SEQ ID NO:32)

mbb2EF-2: GCC GAT GCT ATC CAC CGC CGC (SEQ ID NO: 33)

2253EF-2C: TCT CTT CTT GTT CAA AAC AGA GTA GAT ACC (SEQ ID NO: 34)

Example 2. Spheroplast transformation with the partial fragment of mutated EF2 and ARG4 fragment.

[0106] In the methods of Example 1, there was no selection step against wild type DT. A double transformation was thus employed. First, the mutated EF-2 fragment was transformed into the GS200 strain by electroporation. Then, electroporated cells were cultivated overnight to allow the expression of mutated EF-2 inside cell. Cultivated cells were used for making spheroplasts. The resulting spheroplasts were treated with wild type DT (200 μg/ml) and ARG4 fragment (10 μg) for 1 hour and transformed by $CaCl_2$ and PEG. Only a few transformants of normal colony size were obtained and there was no mutated strain. In addition, there were 100 or more micro-colonies obtained. 100 of these were screened but the mutated strain was not detected.

Example 3. Cloning and sequencing of EF-2 gene from *Pichia pastoris*

[0107] Prior to the cloning of the full sequence of the *Pichia pastoris* EF-2 gene, a partial sequence had been obtained. Initially, the conserved R domain of *Pichia pastoris* EF-2 was amplified from the genomic DNA using two primers derived from the same region of *S. cerevisiae* EF-2 (Perentesis et al., 1992). The 5' primer contained the sequence from position 1933 to 1962 of *S. cerevisiae* EF-2, whereas the 3' primer was complementary to the region of 2227 to 2256. The sequence of 324 nucleotides was then extended towards the 5' end to position 284 and the 3' end to position 2289 in the coding region of *Pichia pastoris* EF-2 gene. The extended sequence was later found to contain several mistakes. To clone the entire *Pichia pastoris* EF-2 gene, two species of cDNA were first synthesized separately from EF-2 mRNA with two different primers. Primer dT22-Not contains a run of 22 T residues complementary to the 3'polyA tail of the mRNA and the recognition sequence for restriction enzyme Not 1. Primer EF-2C2 has 25 nucleotides complementary to nucleotide positions 747 to 771 (Fig. 3 (A) (B) (C) (D)) of the *Pichia pastoris* EF-2 coding sequence. After cDNA synthesis, a homopolymeric track of A residues was added to the 3' end of the cDNA extended from primer C2 by homopolymeric tailing (Sambrook et al., 1989). The 5' end sequence of EF-2 was amplified by PCR from the modified cDNA with EF-2C2 and dT22-Not primers, whereas the 3' end sequence was from the cDNA synthesized

from primers dT22-Not and EF-2C1, which contains 27 nucleotides corresponding to the positions 1927 to 1953. The PCR products representing the 5' end and 3' end sequences of *Pichia pastoris* EF-2 were then separately cloned to pCR2.1-TOPO vector (Invitrogen).

[0108] Five independent clones containing 5' sequence of EF-2 *Pichia pastoris* were selected for sequencing. They were first sequenced with M13 reverse and M13 forward primers located in the vector close to the up and down streams of the insert respectively, and then with an internal prime complementary to the positions 349 to 384 of EF-2 coding sequence. Among the 5 clones, three had identical sequences, one had two different nucleotides at two different internal locations, and the other one had another different internal nucleotides at a different location. These different nucleotides were likely produced by the cloning procedures since none of these different nucleotides were present in the clone derived from genomic DNA. At the 5' end, all five clones also had 57 to 69 nucleotides of the same sequence before the first ATG codon. The largest open reading frame (ORF) of the 5' end sequence starting from the first AUG is 747 nucleotides and the deduced amino acid sequence (249 aa) shares 90% identity with the first 249 aa at the N-terminus of *S. cerevisiae* EF-2. All four clones containing the 3' end sequence of the EF-2 sequence had the same sequence of 675 nucleotides followed by a homopolymeric A track. The largest ORF is 603 nucleotides ended at stop codon TAA, which is 72 nucleotides up stream of the poly-A track. The deduced amino acid sequence (201 aa) shares 85% identity with the last 201 aa at the C-terminus of S. cerevisiae EF-2. Having obtained both the 5' and 3' end sequences of *Pichia pastoris* EF-2, the inventor then designed two primers to amplify the entire the EF-2 gene from the genomic DNA of *Pichia pastoris*. Primer 5'EF-2 is derived from the 5' non-coding region and contains the sequence from positions 28 to 54 relative to the ATG initiation codon. Primer 3'EF- 2C contains 27 nucleotides complementary to positions 2523 to 2549 at the 3' end. After PCR amplification with Pfu polymerase (Stratagene), the PCR products of EF-2 gene were treated with Taq polymerase to have the 3'A-overhangings added (Instruction manual for original TA cloning kit, Invitogen) and then inserted into the TA cloning vector pCR2.1- TOPO. Ten clones were picked, and the restriction enzyme analysis of plasmid DNA isolated from these clones

indicated that they all had the same insert. DNA sequencing was performed first with M13 reverse and M13 forward primers and then advanced step by step towards the opposite ends with primers derived from the sequences obtained from the previous steps. Eight clones were completely sequenced, and found to be identical. The 3' end sequence obtained from the genomic DNA is identical to that from the mRNA. However, compared to the 5'sequence of mRNA, the sequence from the genomic DNA has an insertion of 77 nucleotides in the codon immediately next to the initiation site of the EF-2 ORF (Fig. 2). The insertion has the sequence GTATGT ... CACTAAC ... TAG (SEQ ID NO:35), a conserved pattern of short introns in *S. cerevisiae* (Davis et al., 200; Rymond & Rosbash, 1992). Although introns are common in *S. cerevisiae*, they are rarely present in *Pichia pastoris* (Cregg, personal communication). The coding sequence of *Pichia pastoris* EF-2 is present in Fig. 3 (a) (b) (c) (d). It contains 2526 nucleotides and code for 842 amino acids. The *Pichia pastoris* EF- 2 is the same as the EF-2 of *S. cerevisiae* and *Schizosaccharomyces pombe* in length and shares 88% of identity in amino acid sequence with *S. cerevisiae* (Perentesis et al., 1992) and 78% with *S. pombe* (Mita et al., 1997). Both *S. cerevisiae* and *S. pombe* have two functional EF-2 genes (EFT1 and EFT2) per haploid genome. These two copies of the EF-2 genes encode the same amino acid sequence, but have a few different nucleotides (4 in *S. cerevisiae* and 13 in *S. pombe*) in their coding regions and dissimilar flanking sequences. However, our sequencing data of independent clones derived from mRNA and genomic DNA showed that all of the different clones had the same 5' and 3'end flanking sequences and an identical coding sequence. This plus the evidence of Southern blotting of restriction enzyme digested genomic DNA shows that *Pichia pastoris* has only one copy of the EF-2 gene.

Example 4. Construction of mutating plasmid pBLURA-Δ5'mutEF-2.

[0109] To create DT resistant strains of *Pichia pastoris*, the EF-2 gene was mutated so that the Gly at position 701was changed to an Arg. The strategy employed to introduce the mutation into the genome is based on that described by Shortle et al. (1984) and is shown in Fig. 4. In this method, a truncated form (at only one end) of the targeted gene was used to introduce a mutation to the gene in the genome by

homologous recombination. Integration of the truncated gene fragment bearing a mutation will lead to a situation that the genome contains one intact copy of the gene with the mutation and one truncated copy. Because the targeted site is located close to the 3' end, the inventor used the 5' truncated EF-2 (Δ5'EF-2) as the mutating sequence. Δ5'EF-2 contained 1127 nucleotides from the 3'end of EF-2 starting from position 1432 to 2549 (Fig.3) and was generated by PCR with Pfu polymerase. After cloning into the pCR2.1-TOPO vector, Δ5'EF-2 was mutagenized *in vitro* by oligonucleotide-directed mutagenesis. The mutagenized Δ5'EF- 2 (Δ5'mutEF-2) was then released from pCR2.1-TOPO by restriction enzymes Nhe1 and Not 1 that cut at the 5' and 3' ends respectively, and then cloned into the vector pBLURA-SX (provided by Professor Cregg and described in Geoffrey et al. (2001)) that had been digested by Nhe1 and Not 1. The vector contains the auxotrophic marker URA3. Plasmid DNA pBLURA-Δ5'mutEF-2 purified from bacterial was linearized before being electroporated into the strains of *Pichia pastoris*. The plasmid DNA contains a unique Aat II site located in the EF-2 sequence, about 220 nucleotides before the mutation site. Cleavage at this site will target the plasmid integration to the EF-2 locus and favors the event of the mutagenized sequence being transferred to the intact copy of EF-2. Three uracil auxotrophic strains of *Pichia pastoris* were transformed with the plasmid DNA. They are JC308 (adel arg4 his4 ura3), JC303 (arg4 his4 ura3) and JC307 (his4 ura3), and were all provided by Professor Cregg and described in Geoffrey et al. (2001). JC308 was transformed first followed by JO303 and JC307.

Example 5. Identification of clones containing mutated EF-2.

[0110] After electroporation with the linearized pBLURA-Δ5'mutEF-2 DNA, Cells were spread onto plates containing synthetic complete medium for yeast minus uracil (K.D Medical, Maryland). Ura+ clones were then analyzed by "Colony PCR" for the presence the correct mutations in the intact copy of EF-2. In this method, yeast cells from colonies were picked by tooth pickers and resuspended in 20ul of PCR mix. DNA released from the cells lysed by the first PCR step (94°C for 5 minutes) served as the template for PCR amplification. Five primers were used in the PCR detection procedures: primers 5'EF-2 and 3'EF-2C were described previously in section 4; EF-2

(1318) has the EF-2 sequence from position 1318 to 1341; primer wEF-2 is complementary to the positions 2100 to 2119, whereas primer mEF-2 has the sequence complementary the same positions but specific to the mutations The designed nucleotide mutations shown in Fig.1b created a new Sac II restriction enzyme site that was used to confirm the correct mutations in the genome.

[0111] Primers 5'EF-2 and mEF-2 were first used to detect the mutations in the Ura+ transformants. Fig. 6A shows that 9 (clones 1, 2, 3, 12, 13, 14, 33, 40 and 41) of the 12 selected Ura+ clones of JC308 are mEF-2 primer positive, they had a PCR product of the expected size (about 2.2kbp), whereas clones 25, 38 and 47 were negative. As shown in Fig. 6B, when the same clones were analyzed for the presence of wild type sequence with primers EF-2 (1318) and wEF-2, all three mEF-2- clones were wEF-2 primer positive. A 0.8kbp PCR fragment was produced. All mEF-2 + clones were wEF-2- except for clones 33 and 41 that were also wEF-2+. Finally when primers EF-2 (1318) and 3'EF-2C were used, all of the selected clones yielded a PCR product of about 1.2 kbp as expected (Fig. 6C). The PCR products from the clones that were mEF-2+ and wEF-2- were completely digested by Sac II, whereas those of the clones 25, 38 and 47 that were mEF-2. but wEF-2+ were not cut by the enzyme. In agreement with being both mEF-2+ and wEF-2+, clones 33 and 41 produced both Sac II cleavable and non-cleavable PCR products. To investigate why clones 33 and 41 had both mutated and wild type EF-2, the inventor streaked these clones on new selection plates and let the cells grow to form colonies. The inventor then picked 10 well-isolated colonies from each and performed the PCR with primer EF-2 (1318) plus primer 3'EF-2C and the Sac II digestion steps. None of the colonies had the same mixed PCR products as the originals. PCR products of 4 colonies from clone 33, 7 from 41 were completely digested by Sac II, whereas those of other colonies from clones 33 and 41 were not cut at all. This experiment shows that clones 33 and 44 were each originally formed by two different cells, one had an intact EF-2 with the mutations, and the other had an intact wild type EF-2. The inventor then repeated this experiment and checked some of the clones that had only the Sac II cleavable EF-2 (clones 1, 2, 3, 12, 13, 14, and 40) and confirmed that they only contained the mutated intact EF-2. After the success in obtaining EF-2 mutant clones of JC308, the same selection procedure was

used to identify EF-2 mutant clones of JC303 and JC307. Among the Ura+ positive clones picked for analysis, 35% of them contained only the mutated intact EF-2. This high frequency of complete mutation may be due to the fact that *Pichia pastoris* only has one copy of EF-2 per haploid genome. As shown for CHO cells and *S. cerevisiae*, the Arg substitution for Gly711 of EF-2 in *Pichia pastoris* did not affect cell growth at normal conditions.

Example 6. Expression of DT A chain in the EF-2 mutants.

[0112] To test whether the obtained EF-2 mutants are resistant to DT expression, mutEF2JC307-8, an EF-2 mutant clone (clone 8) of JC307, was transformed with the plasmid DNA of pPIC3-DtA. The construction of pPIC3-DtA was previously described (Woo et al., 2002). Briefly, the DT A chain gene with BamH I at its 5' end and Not I at 3'was amplified by PCR, inserted into *Pichia pastoris* expression vector pPIC 3 (Invitrogen) and digested with these two enzymes. Integration of pPIC3-DtA allows cytosolic expression of DT chain upon methanol induction. This plasmid DNA had previously been used to transform the GS200 strain of *Pichia pastoris* (Invitrogen) and two of the resulting clones (C3 and C4) were used in the study on tolerance of *Pichia pastoris* to DT (Woo at al., 2002). C3 had been characterized as a non-DT A expressing clone, whereas C4 is a DT A expressing clone. After the transformation with pPIC3-DtA, six mutEF2JC307-8, (mutEF2JC307-8- DtA(1) to (6), clones were randomly picked for analysis of their cytosolic expression of DT A chain and their viability after methanol induction. Cells from single colonies of mutEF2JC307-8-DtA(1) to (6), C3 and C4 were grown in 2ml YPD (Yeast extracts-Peptone-Dextose) medium at 30°C overnight before being pelleted down by centrifugation. Cells from each culture were resuspended in YP medium to a density at OD600 nm ± 0.5. Cell suspensions (2 ml) were induced by adding methanol to 1% and incubated at 30°C with vigorous shaking. After methanol induction for 24 hours, cells from 100 µl of each culture were pelleted down and washed with PBS buffer. After this, cells were resuspended in PBS and mixed with protein sample buffer. Finally, the samples were subjected to two cycles of boiling and freezing on dry ice before being analyzed by SDS-PAGE and Western blotting with a DT specific antibody. The cultures of mutEF2JC307-8-DtA(3) and (5),

C3 and C4 were also used for viability assay. This was performed by diluting each culture 104 to 107 fold with PBS buffer, plating 100 µl of aliquot on YPD plate and then counting the colonies appearing on the plates after 3 days incubation at 30°C. The result of SDS-PAGE and Western blotting showed that except for mutEF2JC307-8-DtA(5), all mutEF2JC307-8-DtA clones expressed DT A chain (Fig. 7A). The expression of mutEF2JC307-8-DtA(3) was estimated roughly at 20 µg/ml cell culture. As expected, C3 did not express DT A. Although C4 did express DT A, the protein band was barely visible (Fig. 7B). Before methanol induction, the number of the colony forming units (CFU) per ml of cells was about the same for mutEF2JC307-8- DtA(3) and (5), C3 and C4. After 24 hours methanol induction, the CFU number of mutEF2JC307-8-DtA(3) and (5) and C3 all increased about 103 fold, whereas the CFU number of C4 decreased about 102 (Fig. 8). This result demonstrated that the expression of DT A chain in the cytosol of cells bearing the mutated EF-2 was not toxic to the cells.

Example 7. Test tube culture and small-scale expression in shake-flask culture

[0113] For test tube culture, a single colony or frozen stock was grown in 50 ml of YPD broth in a 250 ml Erlenmeyer flask for 2 days at 30 °C with vigorous shaking, and then the cells were harvested. The cells were resuspended at 8% wet cell density in 3 ml of BMMYC broth (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base without amino acids, 4x10-5% biotin, 0.5% methanol and 1% casamino acids) or appropriate induction broth to give an 8% wet cell density, and induced for 24 hours.

[0114] MutEF2JC307-8 was first used to express the bivalent immunotoxin. Since this EF-2 mutant is auxotrophic for histidine, it was transformed with plasmid pPIC9K containing the final version of the modified gene for the bivalent immunotoxin: A-dmDT390-bisFv described in Woo et al. (2002). Bivalent refers to two repeats of the sFv antibody fragment. The protocols used for transformation, selection for transformants, and protein expression and analysis were described previously (Woo et al., 2002, which is incorporated herein by reference in its entirety for the methods taught threin). After transformation, 12 colonies were randamly picked and analyzed

for protein expression. SDS-PAGE analysis revealed that all of the selected clones expressed the bivalent immunotoxin, although some clones, such as clone number 2 [mutEF2JC307-8(2)], expressed at slightly higher levels than others. When they were cultured and expressed under the same conditions and at the same time, mutEF2JC307-8 (2) expressed the bivalent immunotoxin at the same level as JW102, a clone of GS115 (bearing the wild type EF-2) that had been transformed with pPIC9K-A-dmDT390-bisFv. The expression levels of mutEF2JC307-8(2) and JW102 were about 5 to 10 μg/ml of culture supernatant in shake-tube culture. The fact that mutEF2JC307-8(2) did not yield a higher level of expression demonstrated that other factors in addition to EF-2 ADP ribosylation also limit production of the bivalent immunotoxin.

[0115] In a second attempt to express the bivalent immunotoxin in mutated *Pichia pastoris*, two copies of A-dmDT390-bisFv gene were introduced into mutEF2JC303-5, an EF-2 mutant clone (clone 5) of JC303, which is auxotrophic for histidine and arginine. To build an expression vector with ARG4 selection marker, the inventor cloned the A-dmDT390- bisFv gene (see Fig. 20) into the expression vector pBLARG-SX3 provided by Professor Cregg and described in Geoffrey et al. (2001). This was done by inserting the final version of A-dmDT390-bisFv gene plus the α–factor signal sequence released from pPICZα (Woo et al., 2002) by Hind III and Not I digestion into pBLARG-SX3 that had been cut with these two restriction enzymes. The resulting construct, pBLARG-A-dmDT390-bisFv (Fig 9a), together with pPIC9K-A-dmDT390-bisFv were electroporated at the same time into mutEF2JC303(5). Transformants expressing these two marker genes were selected on plates containing synthetic complete medium minus arginine and histidine (K.D Medical, Maryland). Eighteen colonies were picked from the selection plate and analyzed for their expression of the immunotoxin protein. SDS-PAGE showed that they all secreted roughly the same amount of intact immunotoxin protein into induction media. This amount was similar to that secreted from single copy clones: mutEF2JC307-8(2) and JW102. As shown in Fig. 10A, three of the selected clones (clones 3, 6, 8) also expressed a smaller, but much more abundant protein that reacted with an anti-DT antibody and had the same size as the monovalent immunotoxin (Liu et al., 2000). It seems that the smaller protein is more stable than the intact protein although the

inventor has not yet determined whether this protein was produced from a truncated copy of A-dmDT390-bisFv gene or the proteolytically cleaved product of the intact protein. The figure also shows that there were many other smaller proteins in the culture supernatant that reacted with the anti-DT antibody; they were most likely the proteolytic cleaved products of the intact protein. The smallest and also the most abundant one was characterized as the A chain of DT, which is very stable (Collier 1975) and may account for the final product of proteolytic degradation of the intact protein. The degradation also took place inside the cell (Fig. 10B). Because the A chain is about 1/4 of the size of the intact protein, the amount of the A chain shown on the Western blot indicates that the actual expression level was probably several times higher than the level of intact protein present in the induction medium. A majority of the protein synthesized was probably degraded either before or after secretion out into the medium. Although the double copy clones accumulated the same amount of intact protein in the medium as the single copy clones, the double copy clones produced a larger amount of degraded products, indicating that more gene products had been synthesized. Different measures to control the protein degradation have been employed but the production of the intact protein has not been increased. Thus protein degradation either within or outside the cell is a limiting factor to increase the production of the bivalent immunotoxin. Should this limiting mechanism be overcome or attenuated, it is likely that the mutated EF-2 expression strains will show further increased benefits over non-mutated strains (Microbiol. Biotechnol. 54, 741-50).

Example 8. Previous method of large-scale expression in fermentation culture

[0116] For large scale cultures, the BioFlo 4500 fermentor (New Brunswick Scientific Company), which was installed with a methanol sensor (Raven Biotechnology Company) for precise control of methanol concentration in cultures, was used. The initial fermentation medium (10 L) contained 1% yeast extract, 2% peptone or 2 % soytone , 4% glycerol, 1% casamino acids, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 0.043% PTMI salt solution and 0.01% antifoam 289 (Sigma Co.) or a mixture of antifoam 204 , 0.01 % and Stuktol 0.01 %. Depending on culture conditions, 75% (v/v) glycerol solution having 1.8% PTM1 salt

solution was used for obtaining a desired cell density before methanol induction and/or supplementing an additional carbon source or energy source for methanol induction. 100% methanol solution for induction containing 20 mM PMSF and/or 1.2% PTM1 salt solution was used. Alternatively, induction was performed with a continuous feed of 4:1 methanol/glycerol containing 73 mM PMSF, and PMSF was added to 1 mM final concentration just prior to induction. In order to prepare a seed culture for the fermentor, 50 ml of YPD (1% yeast extract, 2% peptone and 2% glucose) was innoculated with 1 ml of a frozen stock of YYL #8-2 and then cultivated for 2 days at 30°C with vigorous shaking. The 30 ml from the 50 ml culture was used as the first seed culture for inoculating approximately 600 ml of the second seed culture. The DO level in the fermentor was maintained at more than 25% for the whole fermentation run. The pH in the fermentor was kept at 3.5 for growth phase and 7.0 for methanol induction phase. The temperature was set at 28 °C for growth and 15-25 °C for methanol induction. Casamino acids solution (20%) were fed continuously at 20 ml/h during methanol induction or at the maximum speed of a pump for feeding for the first 2 hours of methanol induction. At the temperature of 23 °C for methanol induction, the expression level of the bivalent immunotoxin was the highest among 4 different runs. However, its expression level was similar to that of the current expression strain, JW102. Table 1 summarizes results of 5 fermentation runs.

Table 1. Results of Fermentation Runs

	Run 1 (#27)	Run 2 (#28)	Run 3 (#29)	Run 4 (#36)	Run 5 (#41)
glycerol-fed batch time (hour)	5.5	4	0	7.5	6
cell density at the start of methanol induction (%)	19.44	18.60	11.93	20.02	21.16
final conc. of PMSF (mM)	2[1]	2[1]	2[1]	7[2]	2[1]
casamino acid (g)	100[3]	100[3]	100[3]	100[4]	138[5]
temperature for methanol induction (C)	25	20	15	23	23
methanol consumption (g)	3093	2776	2474	2538	3000
glycerol feeding for methanol induction (g)	475	0	0	0	0
methanol induction time (hour)	43	44	70	44	94
final volume of the supernatant (L)	13.3	12.3	11.9	11.4	13.4
expression level (mg/L) at 22 hours of induction	10	15	10	15	NM[6]
expression level (mg/L) at 42 hours of induction	NM	NM	NM	NM	27.5
expression level (mg/L) at 66 hours of induction	NM	NM	NM	NM	30.0

Expression level (mg/L) at harvest	3.3	18.3	26.6	27.5	32.5
Total amount of the bivalent immunotoxin (mg)	43.9	225.1	316.5	313.5	435.5

1: 50 ml of PMSF solution (3.484 g per 50 ml of methanol) was fed on the based of methanol concentration in the culture for the beginning of methanol induction. After the finish of feeding of PMSF solution, methanol solution containing 12 ml of PTM1 salt solution per 1 liter of methanol was replaced.
2: 15 ml of PMSF solution (1.742 g per 15 ml of methanol) was injected at the beginning of methanol induction. On the basis of methanol concentration, methanol solution (20 mM PMSF and 12 ml of PTMI salt solution / liter of methanol) was fed.
3: 10% casamino acids solution was fed at the maximum speed of a pump at the start of methanol induction.
4: 20% casamino acids solution was continuously fed at 20 ml/hour of pump speed.
5: 15% casamino acids solution was continuously fed at 20 ml/hour of pump speed.
6: not measured

[0117] Under the best conditions tested so far, maximum production of the wild-type expression strain, pJHW #2, is 27.5 mg/L with the total amount of 286.0 mg of the bivalent immunotoxin in 42 hrs of methanol induction. This level could not be increased beyond 42 hrs of induction. However, under conditions adopted from those for pJHW #2, production the EF-2 mutant strain YYL8-2 continued to increase up to 94 hrs after methanol induction in spite the fact that the initial 10L of culture medium was gradually diluted to 13.4L with methanol and 10% casamino acids solution (see run 5). The total amount of the bivalent immunotoxin of run 5 was 435.5 mg (32 mg/L). This is 1.46-fold greater that the maximum production of pJHW #2. The difference in the production of the bivalent immunotoxin between these two strains is reflected by the methanol consumption rates as shown in Fig. 11.

Example 9. Previous method of purification of the bivalent immunotoxin.

[0118] The *Pichia pastoris* supernatant contains materials that compete with A-dmDT390- bisFv in binding to anion exchange resins. In addition, the toxin moiety can not be exposed to pH less than 6.5 without undergoing unfolding of hydrophobic

residues. Therefore a hydrophobic interaction chromatographic step using Butyl-650M (TosoHaas) was employed. This resin preferentially binds monomeric A-dmDT390-bisFv over the dimeric form, a species having greatly diminished biologic activity. The capture step also concentrates a *Pichia pastoris* glycoprotein that appears as a diffuse band of ~40 kD on SDS gels but has the same mobility as A-dmDT390-bisFv under size exclusion chromatography. This material is eliminated by preferentially binding to Con A Sepharose (Pharmacia). A Superdex (Pharmacia) size exclusion step eliminates any A-dmDT390-bisFv dimmer not previously screened during the capture step. The overall yield is 45 % when the fermentation conditions achieve an A-dmDT390-bisFv monomer content of 85 %. The procedure for purification of A-dmDT390-bisFv is presented below:

1. Butyl-650M hydrophobic interaction chromatography
    - Bed volume : 600 ml (in 10 cm diameter column)
    - Flow rate : 50-70 cm/hour
    - sample preparation : solid sodium sulfate and 1 M Tris buffer (pH 8.0) were added to the final concentration of 0.5 M and 20 mM, respectively.
    - sample volume : typically 10 L
    - binding buffer : 500 mM Na2SO4, 1 mM EDTA, 20 mM Tris buffer (pH 8.0)
    - elution buffer : 5% glycerol, 1 mM EDTA, 20 mM Tris buffer (pH 8.0)
    - procedure :
        - o equilibrate the column with binding buffer
        - o applied the sample onto the column
        - o washed with 5 bed volume of binding buffer
        - o eluted A-dmDT390-BisFv with 6 bed volume of elution buffer
        - o regenerated the column by manufacturer's protocol
    - volume of eluted fractions : 3600 ml
2. Diafiltration
    - membrane : Amicon spiral-wound membrane (30 Kd) model S3Y30 (0.23 m2)
    - sample : eluted fractions from capturing step
    - diafiltration buffer : 5% glycerol, 1 mM EDTA, 20 mM Tris buffer (pH 8.0)
    - buffer volume used for diafiltration : 6 volume of the sample
    - pressure : 7 psi
    - final volume : around 2 L
3. Poros 50 HQ ion exchange chromatography
    - Bed volume : 40 ml (in 2.6 cm diameter column)
    - Flow rate : 1 ml/min
    - sample : diafiltrated sample (typically 2 L)
    - binding buffer : 5% glycerol, 20 mM Tris buffer (pH 8.0)
    - elution : 0~500 mM NaCl gradient (10 bed volume) in binding buffer
    - procedure :
        - o equilibrate the column with binding buffer

- o applied the sample onto the column
- o washed with 3 bed volume of binding buffer and started to collect 20 ml of each fraction
- o eluted A-dmDT390-BisFv with 10 bed volume of 0~500 mM NaCl gradient
- o regenerated the column by manufacturer's protocol

• fraction size : 20 ml

4. Con A affinity chromatography
   - • sample : 90~120 ml of the eluted fractions having A-dmDT390-BisFv from Poros IEX
   - • bed volume : 60 ml resin packed in 2.5 cm X 20 cm column
   - • binding buffer : 5% glycerol, 20 mM Tris buffer (pH 8.0)
   - • flow rate : by gravity
   - • procedure
     - o equilibrated the column with binding buffer
     - o applied the sample to the column and started to collect 10 ml of each fraction
     - o added 0.5 M EDTA to each fraction at the final conc. of 1 mM
     - o washed the column with 5 bed volume of binding buffer
     - o regenerated the resin by manufacturer's protocol
5. Superdex 200 prep grade Gel filtration
   - • sample : 50 ml pooled fraction containing A-dmDT390-BisFv from Con A affinity step
   - • sample preparation : 5 M NaCl was added to the final conc. of 200 mM
   - • bed volume : 970 ml of Superdex 200 resin in 5 cm x 60 cm column
   - • buffer : 200 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl (pH 8.0) and 5% glycerol
   - • flow rate : 1 ml/min
   - • procedure
     - o equilibrated the column with binding buffer
     - o applied the sample to the column and started to collect 20 ml of each fraction
     - o eluted the column with 1 bed volume of the buffer
     - o regenerated the resin by manufacturer's protocol

Example 10. Construction of expression vectors pPGAP-Arg and pPGAP-His.

[0119] The promoter for *Pichia pastoris* glyceraldehydes-3-phosphate dehydrogenase gene (P_{GAP}) has been characterized and used for heterologous protein expression in *Pichia pastoris* (Waterham et al., 1997). P_{GAP} is a strong and constitutive promoter. It was reported that protein expression under control of P_{GAP} in glucose-grown *Pichia pastoris* was higher than that of the commonly used P_{AOX1} in methanol-grown cells (Waterham et al., 1997; Döring et al., 1998). The disadvantage of

constitutive promoters in heterologous protein expression is that they are not suitable for proteins that are toxic to the expressing host. Since the EF-2 mutants of *Pichia pastoris* were resistant to cytosolic expression of DT A, these mutants should allow constitutive expression of DT or PE based immunotoxins in their cells. Therefore the inventor used P_{GAP} to drive the expression of A-dmDT390-bisFv in *Pichia pastoris* in the hope that the P_{GAP} would increase the expression level of protein.

[0120] The construct pPGAPArg-A-dmDT390-bisFv was made by replacing the AOX1 promoter of pBLARG-A-dmDT390-bisFv with P_{GAP} (Fig. 9b). First, P_{GAP} was amplified from the expression vector pGAPZ A (Invitrogen) by PCR with primers containing sequences of P_{GAP} 5' and 3' ends. The 5' and 3'end primers had a Nhe I and Hind III added respectively. After digestion with Nhe I and Hind III, the PCR products of P_{GAP} were then inserted in pBLARG-A-dmDT390-bisFv that had been cut with these two restriction enzymes to remove the AOX1 promoter. The construct pPGAPHis-A-dmDT390-bisFv (Fig. 9c) was created by joining DNA fragments from plasmids pPIC9K (Invitrogen) and pPGAPArg-A-dmDT390-bisFv. The plasmid pPIC9K was first cut by Sfu I, after filling in with Klenow Fragment by Not I, then the DNA fragments were separated by agarose gel electrophoresis. The 5.1 kbp fragment containing kanamycin resistant gene, HIS4 gene and 3' AOX1 transcription termination (TT) was isolated and ligated with the plasmid DNA pPGAPArg-A-dmDT390-bisFv that had been digested with Not 1 and Sca I to remove the 3'AOX1 TT and ARG4 gene.

Example 11. Expression of the bivalent immunotoxin under the control of P_{GAP}.

[0121] As done for the expression under AOX1 promoter, one copy clones were obtained by transforming mutEF2JC307-8 with construct pPGAPHis-A-dmDT390-bisFv; two copy clones by transforming mutEF2JC303-5 with both pPGAPArg-A-dmDT390-bisFv and pPGAPHis-A-dmDT390-bisFv. This time the two copy clones were constructed by two steps. First, mutEF2JC303-5 was transformed with pPGAPArg-A-dmDT390-bisFv, after selection and protein expression analysis. The clone that produced the intact immunotoxin at highest level was then transformed with pPGAPHis-A-dmDT390-bisFv.

[0122] Small scale protein expression was carried out by inoculating a single colony to 2 ml YPD, and after overnight growth, cells were seeded in 2 ml expression medium at an OD600 nm ± 0.5, and then incubated at 28°C for 24 hours before the culture supernatant was analyzed for expression of the immunotoxin. The expression medium is the similar to BMMYC used for expression of the immunotoxin under P_{AOX1}, but instead of 0.5% methanol it contains 2% glucose. SDS-PAGE analysis showed that accumulation of the intact protein in the culture supernatant of 2 copy clones was slightly higher than that of 1 copy clones. One of the 2 copy clones (Pgap2-9) has consistently producing 10 to 15 μg of intact protein per ml of culture medium. The results of Western blotting analysis of culture supernatant and extract cell pellet were consistent with those obtain from the expression under P_{AOX1}.

[0123] The production of the bivalent immunotoxin under control of P_{GAP} was slightly higher than that under P_{AOX1} in shake tube culture. Since fermentation allowed cells to grow to very high density, the increase in production under control of P_{GAP} may be more significant when the production is in a bioreactor. The other advantage of P_{GAP} controlled expression is that production procedure wss simpler and shorter. It did not require addition and maintenance of methanol in the expression medium. The whole production procedure was about 40 hours compared to more than 72 hour for that of the P_{AOX1} controlled expression.

Example 12. Yeast strains and strain maintenance.

[0124] In order to optimize fermentation conditions, the inventor used genetically engineered *Pichia pastoris* strain JW102 (formerly named was pJHW #2), which was generated for production of the bivalent immunotoxin (Woo et al., 2002). The AOX1 (alcohol oxidase 1) promoter controlled the expression of immunotoxin by methanol induction. The gene product was secreted by the alpha-prepro leader sequence. To compare the growth profile and fermentation parameters in the fermentor, JW103 (MutS) and X-33 were used (Table 2).

Table 2. Some of the *Pichia pastoris* strains used in this study.

Names	Protein of interest	Phenotypes
JW102*	Secretion of bivalent immunotoxin	His^+ Mut^+
JW103*	Secretion of bivalent immunotoxin	His^+ Mut^S
C-4	Cytosolic expression of A chain of DT	His^+ Mut^+
X-33	Host strain	His^+ Mut^+

* JW102 and JW103 were renamed from pJHW#2 and pJHW#3, respectively (Woo et al., 2002)

[0125] Strain JW102, expressing the bivalent immunotoxin, was genetically very stable. After subculturing the strain more than 60 times onto YPD plates (1% yeast extract, 2% Bacto peptone, 2% dextrose and 2% agar), the strain maintained expression of the bivalent immunotoxin. A colony isolated at the very early stage was expanded in YPD broth (1% yeast extract, 2% Bacto peptone, 2% dextrose) and then kept as frozen stock at –80 °C. Frozen stock was prepared by mixing a 2-day incubation culture with an equal volume of 50% (v/v) glycerol and 1 ml of the mixture was dispensed into a 2 ml Cryo vial.

Example 13. Fermentation.

[0126] The inventor used a BioFlo 4500 fermentor (New Brunswick Scientific Company), which was installed with a methanol sensor (Raven Biotechnology Company) for precise control of methanol concentration in cultures. Methanol consumption rate during methanol induction was measured by weighing a methanol

solution or methanol/glycerol mixed solution every one minute on a computer interfaced balance (PG5002S, Mettler Toledo). The basic initial fermentation medium (10 liters) contained 2% yeast extract, 2% Soytone Peptone (Difco), 4% glycerol, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 0.43% PTM1 salt solution and 0.02% antifoam 289 (Sigma Co.). The PTM1 salt solution consisted of 24.0 mM cupric sulfate, 0.534 mM sodium iodide, 17.8 mM manganese sulfate, 0.827 mM sodium molybdate, 0.323 mM boric acid, 2.1 mM cobalt chloride, 147.0 mM zinc chloride, 234.0 mM ferrous sulfate, 1.64 mM biotin, 188.0 mM sulfuric acid. Depending on the culture conditions, 75% (v/v) glycerol solution having 1.8% PTM1 salt solution was used for obtaining a desired cell density before methanol induction and/or supplementing an additional carbon source during methanol induction. 100% methanol solution during induction containing 10 mM PMSF and/or 1.2% PTM1 salt solution was used. Alternatively, with the EF-2 mutant, the carbon source may be limited to methanol during induction and the methanol feed rate may be limited to about 0.75 ml/min or lower and regulated by a precision pump (Table 3). In run #53, methanol was fully fed by a pump that was controlled by a methanol sensor to maintain a set point of 0.15% methanol in the culture. In run #56, methanol feeding during methanol induction was limited to 0.75 ml/min. Concentration of bivalent immunotoxin in the supernatants taken at various induction time points was determined on Coomassie-stained SDS-polyacrylamide gels. For further comparison between both runs, protein yield of the Butyl 650M HIC capture step was determined from 1 liter of each supernatant. Limited feeding of methanol during methanol induction increased the secretion level of bivalent immunotoxin up to 50 mg/L.

Table 3. Limited feeding of methanol at a rate of 0.75 ml/min during methanol induction increases secretion level of bivalent immunotoxin in the EF-2 mutant strain.

Induction time	Purification step	Run #53 Full feeding of methanol	Run #56 Limited feeding of methanol
22 hr	Supernatant	12.5 mg/L	15.0 mg/L
	Butyl 650M HIC (from 1 L supernatant)	11.7 mg	14.4 mg
44 hr	Supernatant	30.0 mg/L	35.0 mg/L
	Butyl 650M HIC (from 1 L supernatant)	23.4 mg	28.8 mg
67 hr	Supernatant	35.0 mg/L	50.0 mg/L
	Butyl 650M HIC (from 1 L supernatant)	29.3 mg	40.3 mg

[0127] Alternatively, induction was performed with a continuous feed of 4:1 methanol/glycerol containing 10 mM PMSF, and PMSF was added to 1 mM final concentration just prior to induction. In order to prepare a seed culture for the fermentor, 50 ml of YSG broth (1% yeast extract, 2% Soytone Peptone, 1% glycerol) was inoculated with 1 ml of a frozen stock and then cultivated for 2 days at 30 °C with vigorous shaking. 30 ml from a 50 ml culture was used as the first seed culture for inoculating approximately 600 ml of the second seed culture. The DO level in the fermentor was maintained at more than 40% for the whole period of fermentation by O2 supplementation as needed. The pH in the fermentor was kept at 3.5 during the growth phase and 7.0 during the methanol induction phase. The temperature was set at 28 °C for growth and 15~28 °C during methanol induction. A casamino acids or yeast extract solution (10%) was fed continuously at 10 ml/h during methanol induction or at the maximum speed allowable by the feeding pump during the first 2 hours of methanol induction. Reducing the bioreactor agitation may increase the fraction of monomeric and bioactive immunotoxin. A bioreactor agitation of 400 rpm increases the fraction of monomeric and bioactive immunotoxin by 50% over a bioreactor agitation of 800 rpm (Fig. 12). Providing a detergent or other denaturant during agitation may reduce

aggregation of the immunotoxin. Including Tween 20 at 0.01% during agitation of immunotoxin further reduces aggregation and increases the fraction of monomer and bioactive immunotoxin to 90% (Fig. 13). After harvesting the culture, the supernatant was prepared by centrifugation of the culture and then EDTA was added to the supernatant to a final concentration of 5 mM to prevent protein degradation for storage of the supernatant at 4 °C.

Example 14. Purification.

[0128] A scaleable 3-step procedure for purification of the bivalent immunotoxin has been developed (Woo and Neville, 2003) that utilizes borate anion exchange chromatography to eliminate contaminating host glycoproteins. Purifications were performed with 1 L of centrifuged supernatant. No dialysis or diafiltration step was employed. In brief, 1 L of supernatant was mixed with 28.4 g of solid Na_2SO_4 and applied to a 100 ml bed of butyl-650M and eluted with 5% glycerol, 20 mM tris and 1 mM EDTA, pH 8.0, after washing in 200 mM Na_2SO_4. 600 ml of eluant was diluted with 4.2 L of TE buffer (20 mM tris, 1 mM EDTA, pH 8.0) and applied to a 40 ml bed of Poros 50 HQ. The bivalent immunotoxin was eluted in steps of sodium borate buffer from 25-100 mM, and then glycoproteins and some highly aggregated immunotoxin were eluted with 1 M NaCl. 1.2 L of the borate eluant was diluted with 3.6 L of TE buffer and applied to a 5 ml prepacked bed of Hi-trap Q. After washing, the bivalent immunotoxin was eluted with a 0-400 mM NaCl gradient.

[0129] Butyl-650M hydrophobic interaction chromatography (HIC) : Approximately 100 ml of Butyl-650M resin (Tosoh Biosep LLC) was packed in a 5 cm x 20 cm XK column (Amersham Pharmacia Biotech) and the column was equilibrated with Buffer A containing 200 mM Na2SO4, 1 mM EDTA, 20 mM Tris-Cl buffer (pH 8.0). Solid sodium sulfate and 1 M Tris-Cl buffer (pH 8.0) were added to 1 liter of the supernatant to a final concentration of 200 mM and 20 mM, respectively. The sample was filtered with an 802 fluted filter paper (>15 um particle retention: Whatman Inc.; Clifton, NJ, USA) before loading. The flow rate was 44 cm/hour (14.4 ml/min). After equilibrating the column, 1 L of the prepared sample was applied onto the column, and then the column was washed with 6 column volumes of binding buffer A. The bound

proteins to Butyl-650M resin were eluted with 6 column volumes of Buffer B containing 5% glycerol, 1 mM EDTA, 20 mM Tris-Cl buffer (pH 8.0). The eluted fractions having the immunotoxin were pooled for the next step (volume: ~600 ml). After each run, the column was regenerated according to the manufacturer's protocol. All steps were performed in a cold room except for the first step that was carried out at room temperature.

[0130] Poros 50 HQ anion exchange chromatography (AEX) by step-elution with sodium borate buffer: Approximately 40 ml of Poros 50 HQ resin (PerSeptive Biosystems) was packed in a 2.6 cm x 20 cm XK column (Amersham Pharmacia Biotech) and then the column was equilibrated with Buffer B. The pooled sample from the previous step was diluted with 4.2 L of TE buffer (20 mM Tris-Cl, 1 mM EDTA, pH 8.0). The diluted sample was loaded onto the column at a flow rate of 80.2 cm/hour (7.08 ml/min), and then the column was washed with 6 column volumes of Buffer B. The bound proteins were eluted in steps of sodium borate of 25 mM, 50 mM, 75 mM and 100 mM in Buffer B (10 column volumes for each step). These eluted fractions were pooled for the next step. The residual protein bound to the resin was stripped with 6 column volumes of 1 M NaCl in Buffer B. After each run, the column was washed with 0.5 M NaOH and then re-equilibrated with Buffer B minus 5% glycerol for the next use.

[0131] Hi-trap Q anion exchange chromatography: A prepacked Hi-trap Q anion exchange column (5 ml) was purchased from Amersham Pharmacia Biotech. The pooled sample from the previous step was diluted with 3.6 L of TE buffer. The sample was loaded onto the equilibrated column with Buffer B at a flow rate of 221.5 cm/hour (7.08 ml/min). The column was washed with 5 column volumes of Buffer B. The bound immunotoxin was eluted with a linear 0~400 mM NaCl gradient in Buffer B (20 column volume). The flow rate for washing and eluting steps was 2 ml/min and fraction size was 5 ml.

Example 15. SDS-PAGE and Western blotting.

[0132] Proteins in culture supernatants were subjected to SDS-PAGE utilizing tris-glycine 4~20% precast gels (Invitrogen) under non-reducing and/or reducing conditions. For Western blotting, the fractionated proteins were transferred onto nitrocellulose membranes by electroblotting. Non-specific binding was blocked with 5% nonfat skimmed milk in TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). Goat polyclonal antibody directed against diphtheria toxin (Thompson et al., 1995) diluted 1:2000 was used as the primary antibody, and alkaline phosphatase-conjugated rabbit anti-goat IgG (Roche Molecular Biochemicals) diluted 1:5000 was used as the secondary antibody. The immunotoxin was visualized with one-step NBT/BCIP substrate (Pierce Chemical Company).

Example 16. Cytotoxicity assay.

[0133] The tests to measure the specific cytotoxicity of anti-human anti-CD3 immunotoxins expressed in *Pichia pastoris* were performed as described (Neville et al., 1992). Briefly, immunotoxins were applied to Jurkat cells, a human CD3ε+ T cell leukemia line, (5×10^4 cells/well) in 96-well plates in leucine-free RPMI 1640 medium. After 20 hours, a 1 hour pulse of [^{3}H] leucine was given. The cells were then collected onto filters with a cell harvester. After addition of scintillant, samples were counted in a Beckman scintillation counter using standard liquid scintillation counting techniques.

Example 17. Measurement of cell viability.

[0134] In order to measure cell viability of cultures taken at various time points in fermentation, Ormerod's method was modified (Ormerod, 2000). Fluorescein diacetate (FDA) and propidium iodide (PI) were used as vital dyes of cell viability. FDA taken up by *Pichia pastoris* was converted to fluorescein by an intracellular esterase. If a cell has an intact plasma membrane, fluorescein is retained and PI is excluded. In brief 500 µl of a suspension of *Pichia pastoris* cells at 106 cells/ml in the PBS buffer were mixed with 50 µl of FDA solution (10 µg/ml) and 50 µl of PI solution (100 µg/ml). After incubation at room temperature for 10 min, cell viability of the sample was analyzed by

flow cytometry. The viable cell gate included green fluorescence and excluded red fluorescence.

Example 18. Analytic gel filtration.

[0135] A Superdex 200 10/300 GL prepacked column (dimension 1.0 cm x 30 cm) was purchased from Amersham Pharmacia Biotech. The column was connected to an HPLC system (GBC Scientific Equipment; Arlington Heights, IL, USA). Gel filtration buffer consisted of 90 mM sodium sulfate, 10 mM sodium phosphate monobasic and 1 mM EDTA (pH 8.0). The flow rate was 0.5 ml/min and injection volume was 500 µl.

Example 19. Immunotoxin toxicity during expression in *Pichia pastoris* is manifest by a reduction in AOX1 activity.

[0136] The bivalent immunotoxin in *Pichia pastoris* was expressed via the secretory route. This secretion of the bivalent immunotoxin in *Pichia pastoris* significantly attenuated the toxicity of the immunotoxin (Woo et al., 2002), but the bivalent immunotoxin expression depressed metabolic capacity of methanol utilization and growth reduction during methanol induction in fermentor culture.

[0137] In the metabolism of methanol by *Pichia pastoris*, oxidation of methanol by alcohol oxidase (AOX1) is the rate-limiting reaction (Veenhuis et al., 1983), and the amount of the AOX1 gene product determines how rapidly methanol is metabolized. AOX1 can account for 30% of the proteins in *Pichia pastoris* cells utilizing methanol. Therefore, measurement of methanol consumption rates during methanol induction reflects the AOX1 level and provides an indication of how the expression of the bivalent immunotoxin affects protein synthesis and degradation of AOX1 in *Pichia pastoris*. To this end, profiles of the methanol consumption rate in a fermentor culture were compared between the wild type host strain X-33 and the JW102 strain, which expressed the bivalent immunotoxin via the secretory route. Under the fermentation conditions where casamino acid supplements were used during methanol induction, X-33 had a maximum 1.95 ml/min of methanol consumption at 25 °C and the consumption rate was maintained at more than 70% of the maximum rate during the

whole methanol induction phase (Fig. 11). For the immunotoxin expressing strain JW102 (Mut+), the maximum methanol consumption rate was approximately 1.10 ml/min at 23 °C. After the peak point at 7~8 hours following the initiation of methanol induction, the consumption rate was gradually decreased to 20% of the maximum rate. Within the first 18 hours of methanol induction, the methanol consumption rate dropped below 50% of maximum methanol consumption rate (Fig. 11). These low levels of methanol consumption were associated with low levels of wet cell density increase, 2% for JW102 versus 10.5% for X-33 at 44 hours (Fig. 14).

Example 20. Use of PMSF and casamino acids or yeast extract during the methanol induction phase

[0138] In the initial stages of fermentation optimization, supplementing of PMSF and casamino acids during methanol induction was crucial for boosting the expression level in the fermentor. Without these two components during methanol induction, the expression level of the bivalent immunotoxin reached a maximum 7 hours after initiation of methanol induction and then decreased. However, supplementing these two components during methanol induction extended the optimal induction time from 7~8 hours to 24~48 hours after the start of methanol induction. In addition, the expression level was improved up to 2-fold.

[0139] To avoid the use of animal-derived material, the inventor substituted yeast extract for casamino acids. This change resulted in a substantial increase in the expression level by 30% and in wet cell density by 45%. Gain of wet cell density for JW102 by continuous feeding of yeast extract was close to that for X-33 during methanol induction (Fig. 14). These improvements were due to constancy of the methanol consumption rate at greater than 80% of the maximum rate (Fig. 11).

Example 21. Use of methanol/glycerol mixed feed during methanol induction.

[0140] The expression level of the bivalent immunotoxin was positively related to the gain of wet cell density during the first 44 hours of methanol induction. In low-producing cultures, the gain of wet cell density was less than 6.0%. However, in fermentation runs producing more than 25 mg/L of the bivalent immunotoxin, the gain

of wet cell density (%) during the first 44 hours of methanol induction was an average of 9.26% (Fig. 14). The gain of wet cell density during methanol induction was hard to achieve without continuous feeding of glycerol as the additional carbon source. Therefore a methanol/glycerol (4:1) mixed feed was used to support cell growth during methanol induction.

Example 22. Low temperature improves secretion of bivalent immunotoxin.

[0141] Lowering the temperature during methanol induction phase improved the expression level of the bivalent immunotoxin. To determine the effect of low temperature on the secretion of bivalent immunotoxin in *Pichia pastoris*, various temperatures ranging from 28 °C to 15 °C were employed during methanol induction. At temperatures from 28 °C to 23 °C, similar expression levels were observed. However, the expression level was improved almost 2-fold at a temperature below 20 °C down to 15 °C. The highest expression level was observed at 17 °C (Fig. 15A), but the final yield obtained by purification of the immunotoxin was the highest at 15 °C (Fig. 15B). In addition, the supernatant taken at 67 hours of methanol induction shows the highest expression level and highest yield at 15 °C. Low temperature at 15 °C suppressed protease activity in the cultures because the expression level of the bivalent immunotoxin was not changed even in the absence of PMSF. Lowering the temperature during methanol induction reduced methanol consumption as shown in Fig. 15C.

Example 23. Complex media for production of bivalent immunotoxin in *Pichia pastoris*.

[0142] The uses of the complex components in the initial fermentation media were necessary to obtain a reasonable range of the expression level of the bivalent immunotoxin in the fermentor. In the initial fermentation runs, very low production of the bivalent immunotoxin in the fermentor was observed when the standard defined medium was used. Therefore, the inventor developed Soytone Peptone and yeast extract-based medium containing 4% glycerol, 2% yeast extract, 2% Soytone Peptone,

1.34% yeast nitrogen base with ammonium sulfate without amino acids, 0.435% PTM1 salts solution and 0.01% antifoam 289.

Example 24. Mut+ versus MutS phenotype.

[0143] Different Mut (methanol utilization) phenotype strains derived from *Pichia pastoris* GS115 (Mut+) and KM71 (MutS) were tested to compare the expression level of the bivalent immunotoxin in the fermentor. In the fermentor, the MutS phenotype strain has advantages, such as easy control of induction temperature, no need to supply pure oxygen, and resistance to a high concentration of methanol. Although these two different phenotype strains did not make a difference in the expression level in test tube culture, the expression level of the Mut+ strain in the fermentor was 5~7-fold higher than that of the MutS strain.

Example 25. pH shifting procedure reduces contaminant proteins in the supernatant.

[0144] There was a great difference between shake flask culture and fermentor culture for the expression of the bivalent immunotoxin. In shake flask culture, it is possible to replace the culture medium with fresh induction medium, resulting in removal of cell membrane fragments, DNA and proteases derived from cell lysis during the growth period and proteins secreted by *Pichia pastoris*. However, those molecules accumulate for the whole period of fermentation and they are often problematic in the purification process.

[0145] In order to reduce this kind of problem in the fermentor, a pH shifting procedure was employed. *Pichia pastoris* can normally grow within the range of pH 3~7. *Pichia pastoris* was cultivated at a low pH such as pH 3.5 during the glycerol batch phase and the glycerol-fed batch phase, and induced at pH 7.0 for production of the bivalent immunotoxin. The pH shifting procedure provided the supernatant with the dominant bivalent immunotoxin, because the amount of secreted proteins in *Pichia pastoris* was significantly decreased at low pH even though the expression level of the bivalent immunotoxin was not affected.

Example 26. The use of glucose for tight control of the AOX1 promoter.

[0146] In general, tight gene control is necessary to obtain toxic proteins in host cells. The expression of the bivalent immunotoxin was toxic to *Pichia pastoris*. Since the AOX1 promoter cannot tightly control gene expression in the presence of glycerol as the carbon source, the bivalent immunotoxin was observed before methanol induction on Coomassie stained SDS-polyacrylamide gels. The glycerol-fed batch phase was replaced with a glucose-fed batch phase for tight gene control, because glucose represses AOX1-driven gene expression (Tschopp et al., 1987). However, the replacement of glycerol with glucose in the fed batch phase did not change the final expression level of the bivalent immunotoxin. Glycerol was used during the fed batch phase because glucose took time to dissolve at a high concentration. When combined with the glycerol-fed batch phase, the pH shifting procedure prevented the appearance of the bivalent immunotoxin on Coomassie-stained SDS-polyacrylamide gels during the glycerol-fed batch.

Example 27. Optimal pH for expression of the bivalent immunotoxin.

[0147] In order to determine optimal pH for the expression of the bivalent immunotoxin, the expression strain JW102 was induced for 24 hours in the range of pH 3.5 to 8.0 in test tube cultures, and the bivalent immunotoxin in the supernatants was compared on a Coomassie-stained SDS-polyacrylamide gel and Western blotting. Sodium citrate buffer (pH 3.5~5.5), bis-tris buffer (pH 6.0~7.0) and tris buffer (pH 7.5~8.0) were used for maintenance of the cultures at the indicated pH. Simultaneously, colony forming units in the cultures at the end of methanol induction were measured as previously described (Woo et al., 2002). Below pH 6.0, the bivalent immunotoxin was not detectable on Western blots. Although the Western blot shows similar expression levels in the range of pH 6 to 8, the Coomassie-stained SDS-polyacrylamide gel indicates pH 7.0 was the optimum pH level for the expression of the bivalent immunotoxin. *Pichia pastoris* had similar colony forming units in the range of pH 3.5 to 7.0, but the colony forming units were sharply decreased at above pH 7.0. Since pH 7.0 was the upper edge of optimal pH range, the expression level at pH 6.8

was also tested in the fermentor. However, there was no difference in the expression level at pH 6.8 and 7.0.

Example 28. Reproducibility and cell viability of optimized fermentation runs.

[0148] Under the optimized fermentation conditions, the expression level of the bivalent immunotoxin increased to 40 mg/L at 67 hours of methanol induction (Fig. 16). The expression levels in the supernatants at 44 hours and 67 hours of methanol induction, and the final yield obtained by the 3-step purification procedure for the bivalent immunotoxin were reproducible. Prior to optimizing the fermentation conditions, the inventor observed a variation of expression level from batch to batch. As shown in Table 4, very similar levels of bivalent immunotoxin were obtained in 3 independent fermentation runs under the optimized conditions. More importantly, the final yields of the purified bivalent immunotoxin were very similar to each other, indicating that produced supernatants had similar quality of the bivalent immunotoxin. Under the optimized fermentation conditions, cell viability during methanol induction phase was maintained at greater than 95% as determined by flow cytometry.

Table 4. Reproducibility of optimized fermentation condition[1] and purification[2].

Run no.	Methanol induction time (hrs)	Expression level (mg/L)	Purified immunotoxin from 1 L supernatant (mg)
1	44	30	16
	67	40	18
2	44	30	16
	67	40	18
3	44	30	16
	67	40	18

[1] Optimized condition: induction temperature at 15 °C; continuous feeding of 10% yeast extract feeding at 8.95 ml/hr; methanol/glycerol (4:1) mixed feed for methanol induction.

[2] For purification of the bivalent immunotoxin, a 3-step procedure (Woo and Neville, 2003) was used.

Example 29. Relationship between induction time and formation of the aggregates.

[0149] Immunotoxin aggregates were accumulated in the supernatant during induction. In order to determine the relationship between induction time and aggregate formation, fractionated samples were taken at 22, 44 and 67 hours of methanol induction by a Superdex 200 gel filtration and then analyzed fractionated samples on SDS-PAGE gels. The 22, 44 and 67 hour samples contained 50.0, 60.0 and 66.7% of dimeric and higher oligomeric forms of the immunotoxin. These aggregate forms of

the immunotoxin had only 10% specific toxicity of the monomeric immunotoxin to Jurkat cells.

[0150] In addition, the accumulation of immunotoxin aggregates significantly reduced bioactivity of the supernatant. However, bioactivity was recovered by the butyl 650M capturing step developed in a previous study (Woo and Neville, 2003). This result suggested the possibility that some portion of immunotoxin aggregates were reversible.

[0151] The use of antifoam agents at a concentration above 0.01% reduced formation of aggregates. These immunotoxin aggregates did not bind well in thiophilic adsorption used as the capture step before developing a 3-step purification procedure (Woo and Neville, 2003). In the initial stages of fermentation optimization, antifoam agents were used at the minimum concentration that could control excessive foaming in the fermentor. However, more than 50% of the bivalent immunotoxin was lost at the first capturing step when antifoam 289 was used at 0.005% in the initial fermentation medium. The use of antifoam 289 at a concentration of more than 0.01% in the initial fermentation medium was crucial to obtain reasonable yields of more than 90% in the first capture step.

Example 30. Protein Quantification by Comparison on SDS-PAGE and Cytotoxicity Assay.

[0152] The concentration of the immunotoxin was quantified by SDS-PAGE using an immunotoxin standard of known concentration prepared previously (Woo et al., 2002). Samples to be measured were subjected to SDS-PAGE utilizing tris-glycine 4-20% precast gels (Invitrogen) under non-reducing or reducing conditions.

[0153] The specific cytotoxicity of the purified anti-human anti-CD3 immunotoxins were performed as described (Neville et al., 1992). Briefly, immunotoxins were applied to Jurkat cells, a human CD3ε+ T cell leukemia line, (5 x 10^4 cells/well) in 96-well plates in leucine-free RPMI 1640 medium. After 20 hours, a 1 hour pulse of [^{3}H] leucine was given. Cells were collected onto filters with a Skatron

harvester. After addition of scintillant, samples were counted in a Beckman scintillation counter using standard LSC techniques.

Example 31. Butyl 650M hydrophobic interaction chromatography (Butyl 650M HIC).

[0154] As shown in Fig. 17, Butyl 650M HIC was an efficient capture step for immunotoxin in supernatant. However, glycoproteins were also purified with the immunotoxin during this step. Among these glycoproteins, identified by periodic acid Schiff staining, the glycoprotein species of approximately 45 kDa (arrow in Fig. 17) impeded isolation of the pure immunotoxin. By conventional chromatography such as gel filtration and anion exchange chromatography, these glycoproteins were not separated from the immunotoxin, indicating that these 45 kDa glycoprotein species were present in dimeric form and had similar isoelectric points. Therefore these 45 kDa glycoproteins were very similar to the immunotoxin in size and isoelectric point as well as in hydrophobicity.

[0155] Various hydrophobic resins which complied with GMPs (Good Manufacturing Practices) were evaluated. Among these resins, Butyl 650M appeared to have the best binding and eluting profile of the immunotoxin. Other hydrophobic resins may be used in the present invention. The inventor also found that 200 mM of sodium sulfate was a suitable concentration for binding of the immunotoxin to the butyl 650M resin.

[0156] The fermentor culture normally had approximately 30% of wet cell density at the end of the fermentation run. In large-scale production, the supernatant is obtained by continuous centrifugation requiring a 3-fold dilution of the high cell density culture. The immunotoxin in the diluted sample was processed the same as the immunotoxin in the supernatant which was effectively bound to the Butyl 650M resin at 200 mM sodium sulfate.

Example 32. Poros 50 HQ anion exchange chromatography by step-eluting with sodium borate buffer.

[0157] By employing borate anion exchange chromatography, the immunotoxin was successfully separated from the *Pichia pastoris* glycoproteins (Fig. 18). The immunotoxin was bound to anion resin by diluting the sample from the previous step, simplifying the purification procedure. In fractions eluted with 50 mM, 75 mM and 100 mM sodium borate in Buffer B (lane 9, 10,11 in Fig. 18), most of the immunotoxin was present in monomeric form. These 3 fractions were pooled for the next step.

[0158] In order to remove glycoprotein species in the sample obtained from the previous step, sodium borate in anion exchange chromatography was used, because sodium borate increases the negative charge of glycoproteins by binding to the carbohydrate residues of the glycoproteins. The immunotoxin binds to anion exchange resins at pH 8.0(Woo et al., 2002). Preliminary experiments were designed for optimizing binding conditions of the immunotoxin in the presence of sodium borate. Aliquots of the dialyzed sample against Buffer B were mixed with different volumes of 200 mM sodium borate in Buffer B to obtain the designated concentration of sodium borate. The prepared samples were then loaded onto a Poros 50 HQ anion column (40 ml) equilibrated with Buffer B containing a corresponding concentration of sodium borate. At 100 mM of sodium borate the immunotoxin did not bind to the Poros 50 HQ anion resin, but the majority of glycoproteins still bound. At a concentration of sodium borate below 50 mM, the immunotoxin bound to the Poros 50 HQ anion resin.

[0159] Conditions of step elution were further analyzed with sodium borate after binding of the immunotoxin to an anion exchange column. First, the sample dialyzed against Buffer B was bound to the anion column and then eluted in steps of increasing concentration of sodium borate (100, 120, 140, 200 mM) and 1 M NaCl. The bound immunotoxin was mainly eluted at 100 mM sodium borate, but these eluted fractions also contained significant amounts of 45 kDa glycoproteins which were not separable in the next step. The majority of glycoproteins were eluted at 1 M NaCl. After loading the same sample as the first experiment, the bound immunotoxin was eluted in steps of 50, 75 and 100 mM sodium borate and 1 M NaCl. A majority of the bound

immunotoxin was eluted at 75 mM sodium borate. However, a protein band corresponding to 21 kDa was included in the fraction eluted with 50 mM sodium borate. After binding to the column, the bound immunotoxin was eluted in steps of 25, 50, 75 and 100 mM sodium borate and 1 M NaCl in Buffer B (Fig. 18). By washing with 25 mM sodium borate buffer, the amount of a protein band corresponding to 21 kDa was reduced.

Example 33. Comparison with phenylboronate affinity chromatography.

[0160] In order to compare separation profiles, phenylboronate affinity chromatography was performed. The eluant from the butyl 650M HIC capture step was dialyzed against the low ionic strength buffer (10 mM HEPES, 0.25 mM EDTA and 20 mM MgCl2, pH 8.2) for phenylboronate affinity chromatography. The dialysed sample was applied to a 5 ml bed volume column of phenylboronate agarose (Sigma Co.), washed with the same buffer, and then the bound proteins were eluted with either 0-100 mM sodium borate gradient or 0-50 mM sorbitol gradient in the same buffer (20 bed volumes). Glycoproteins and the immunotoxin were bound under binding condition of low ionic strength. The glycoproteins and immunotoxin were not separated by phenylboronate affinity chromatography. The glycoproteins and immunotoxin were co-eluted with either 0-100 mM sodium borate gradient or 0-50 mM sorbitol gradient.

Example 34. Q anion exchange chromatography.

[0161] Q anion exchange chromatography was used for concentration of the diluted sample that was obtained from the Poros 50 HQ anion exchange chromatography. At a concentration of sodium borate below 50 mM the immunotoxin was bound to the anion exchange resin. Accordingly, the pooled sample from the previous step was diluted with 3 sample volumes of TE buffer (20 mM Tris-Cl and 1 mM EDTA, pH 8.0), resulting in less than 20 mM of sodium borate in the diluted sample. As expected, the immunotoxin was effectively bound to the Q anion exchange column. The bound immunotoxin was eluted with 0~400 mM NaCl gradient elution (20 column volumes). The immunotoxin fractions were pooled and then assessed for

yield, purity and toxicity of the final preparation by SDS-PAGE and protein synthesis assay.

Example 35. Protein Yield, Repeatability of Purification Procedure, Purity and Function of the Purified Immunotoxin.

[0162] Table 5 summarizes the immunotoxin yields which were obtained in 3 batches of the 3-step purification runs by using the supernatants taken at 44 hours of methanol induction from 3 fermentation runs which were carried out under relatively similar fermentation conditions and had similar expression levels of the immunotoxin. The average yield of this purification batch was 52.8%. By using the 3-step purification procedure, the inventor was able to obtain approximately 16 mg of the purified material from 1 liter of supernatant. The starting supernatants had different levels of immunotoxin aggregates and monomeric immunotoxin depending on the induction time during fermentation run. Among these immunotoxin aggregates, some portions could be reversible to monomeric form of the immunotoxin during the Butyl 650M HIC step. Fractionation of supernatant by gel filtration and subsequent SDS-PAGE analysis showed that the supernatants contained more than 50% of the immunotoxin aggregates. However, the final yield of the immunotoxin after the 3-step purification procedure was 52.8%, indicating that a portion of the aggregates could be dissociated into monomeric immunotoxin during purification.

[0163] A comparison of our purification procedure applied to 3 separate fermentation runs that contained similar amounts of supernatant immunotoxin demonstrates good repeatability of the procedure with respect to yields (Table 4).

[0164] The purity of the purified immunotoxin was assessed by analytical gel filtration. The immunotoxin in the final preparation displayed a single peak corresponding to the monomeric form of the immunotoxin (Fig. 19A). The analyses of purity of the final preparations confirmed that the 3-step purification yielded an immunotoxin with ~ 98.0% purity (Fig. 19B).

[0165] To investigate the effects of the 3-step purification procedure on immunotoxin bioactivity, a protein synthesis assay for the specific T cell toxicity of the

final preparation was performed. The estimated concentration of the immunotoxin in the final preparation coincided with concentration of the immunotoxin standard.

Table 5. Comparison of immunotoxin purification from *Pichia pastoris* fermentor cultures*.

Batch no.	step	IT conc (ug/ml)	volume (ml)	total IT (mg)	yield (%)	acc. yield (%)
1	Supernatant	30:0	1000	30.0	100.0	100.0
	Butyl 650M HIC	45.0	585	26.3	87.8	87.8
	Poros 50 HQ borate AEX	15.0	1200	18.0	67.0	60.0
	Q AEX	400.0	40	16.0	88.9	53.3
2	Supernatant	30.0	1000	30.0	100.0	100.0
	Butyl 650M HIC	40.0	585	23.4	78.0	78.0
	Poros 50 HQ borate AEX	15.0	1200	17.5	74.6	58.2
	Q AEX	400.0	40	16.0	91.6	53.3
3	Supernatant	30.0	1000	30.0	100.0	100.0
	Butyl 650M HIC	40.0	585	23.4	78.0	78.0
	Poros 50 HQ borate AEX	15.0	1200	18.0	67.0	60.0
	Q AEX	450.0	35	15.8	87.5	52.5

* IT, immunotoxin; acc., accumulated; HIC, hydrophobic interaction chromatography; AEX, anion exchange chromatography. Supernatants were obtained from 3 fermentation runs at 44 hours of methanol induction.

[0166] Throughout this application various publications are referenced. Full citations for these publications are as follow. Such publications mentioned are hereby incorporated in their entirety by reference in order to more fully describe the state of the art to which this invention pertains.

[0167] It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those

skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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CLAIMS

What is claimed is:

1. A method of expressing an immunotoxin in *Pichia pastoris* comprising

   a) growing the *Pichia pastoris* in a growth medium comprising an enzymatic digest of protein and yeast extract; and

   b) performing methanol induction of the *Pichia pastoris* with a methanol and glycerol containing feed, wherein the *Pichia pastoris* is contacted with a phenylmethanesulfonyl fluoride and a source of amino acids and wherein the methanol induction is at a temperature of below about 17.5°C.

2. The method of claim 1 wherein the immunotoxin is a fusion protein.

3. The method of claim 1 wherein the immunotoxin comprises a diphtheria toxin moiety.

4. The method of claim 3 wherein the diphtheria toxin moiety is truncated.

5. The method of claim 4 further comprising a CD3 antibody moiety.

6. The method of claim 5 wherein the immunotoxin comprises A-dmDT390-bisFv(G_4S).

7. The method of claim 1 wherein the *Pichia pastoris* comprises a mutation in the amino acid sequence encoding EF-2.

8. The method of claim 1 wherein the enzymatic digest of protein is an enzymatic digest of soy protein.

9. The method of claim 1 wherein the ratio of methanol to glycerol in the methanol and glycerol containing feed is about 4:1.

10. The method of claim 1 wherein the source of amino acids is a yeast extract.

11. The method of claim 1 wherein the *Pichia pastoris* is contacted with the phenylmethanesulfonyl fluoride and the source of amino acids for at least 2 hours.

12. The method of claim 1 wherein the phenylmethanesulfonyl fluoride is dissolved in the 4:1 methanol glycerol induction feed and the concentration does not exceed 10 mM.

13. The method of claim 1 wherein the temperature is about 15°C.

14. The method of claim 1 wherein the composition of the growth medium is about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate without amino acids, and about 0.435% PTM1 solution.

15. The method of claim 14 wherein the growth medium further comprises an antifoaming agent.

16. The method of claim 15 wherein the antifoaming agent is at a concentration of about 0.01% or greater.

17. The method of claim 16 wherein the composition of the growth medium is about 4% glycerol, about 2% yeast extract, about 2% enzymatic digest of soy protein, about 1.34% yeast nitrogen base with ammonium sulfate without amino acids, about 0.435% PTM1 solution and about 0.01% antifoaming agent.

18. The method of claim 1 wherein the dissolved oxygen concentration is maintained at a value of 40% or higher.

19. The method of claim 1 wherein the growth step is at a pH of about 3.5 and the methanol induction step is at a pH of about 7.0.

20. The method of claim 1 further comprising a bioreactor agitation of about 400 rpm.

21. The method of claim 1 further comprising adding a denaturant at about 0.01% or greater.

22. A method of expressing an immunotoxin in *Pichia pastoris* with a mutation in the amino acid sequence encoding EF-2 comprising

    c) growing the *Pichia pastoris* with a mutation in the amino acid sequence encoding EF-2 in a growth medium comprising an enzymatic digest of protein and yeast extract; and

    d) performing methanol induction of the *Pichia pastoris* with a mutation in the amino acid sequence encoding EF-2 with a methanol containing feed, wherein the *Pichia pastoris* with a mutation in the amino acid sequence encoding EF-2 is contacted with a phenylmethanesulfonyl fluoride and a source of amino acids and wherein the methanol induction is at a temperature of below about 17.5°C.

23. The method of claim 22 wherein the methanol containing feed is limited to a rate of about 0.75 ml/min.

24. The method of claim 22 further comprising a bioreactor agitation of about 400 rpm.

25. The method of claim 22 further comprising adding a denaturant at about 0.01% or greater.

26. A method of purifying a non-glycosylated immunotoxin comprising

    a) loading a solution containing the non-glycosylated immunotoxin onto a hydrophobic interaction column;

    b) obtaining a first non-glycosylated immunotoxin containing eluant from the hydrophobic interaction column;

    c) loading the non-glycosylated immunotoxin containing eluant from step (b) onto an anion exchange column;

d) obtaining a second non-glycosylated immunotoxin containing eluant from the anion exchange column by eluting the non-glycosylated immunotoxin with a sodium borate solution;

e) diluting the concentration of sodium borate in the second non-glycosylated immunotoxin containing eluant from step (d) to about 50 mM or less;

f) concentrating the diluted non-glycosylated immunotoxin containing eluant from step (e) over an anion exchange column; and

g) obtaining a purified non-glycosylated immunotoxin from the anion exchange column.

27. The method of claim 26 wherein the non-glycosylated immunotoxin is expressed in yeast.

28. The method of claim 27 wherein the yeast is *Pichia pastoris*.

29. The method of claim 26 wherein the immunotoxin is a fusion protein.

30. The method of claim 26 wherein the immunotoxin comprises a diphtheria toxin moiety.

31. The method of claim 30 wherein the diphtheria toxin moiety is truncated.

32. The method of claim 31 further comprising a CD3 antibody moiety.

33. The method of claim 32 wherein the non-glycosylated immunotoxin comprises A-dmDT390-bisFv(G_4S).

34. The method of claim 26 further comprising washing the anion exchange column with about 25 mM sodium borate solution prior to eluting with the sodium borate solution.

35. The method of claim 26 wherein the concentration of the sodium borate solution in step (d) is between about 50 mM and about 200 mM.

36. The method of claim 35 wherein the concentration of the sodium borate solution in step (d) is between about 75 mM and about 100 mM.

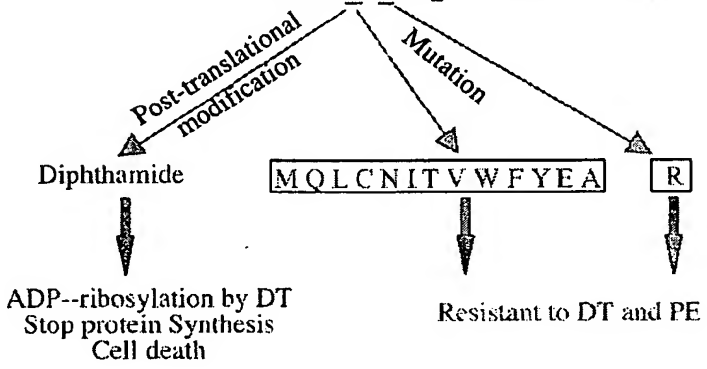
37. The method of claim 26 wherein the concentration of sodium borate in step (e) is about 20 mM.

Abstract

In one aspect, the present invention relates to a method of expressing an immunotoxin in *Pichia pastoris* comprising a) growing the *Pichia pastoris* in a growth medium comprising an enzymatic digest of protein and yeast extract; and b) performing methanol induction of the *Pichia pastoris* with a methanol and glycerol containing feed, wherein the *Pichia pastoris* is contacted with a phenylmethanesulfonyl fluoride and a source of amino acids and wherein the methanol induction is at a temperature of below about 17.5°C.

In another aspect, the present invention relates to a method of purifying a non-glycosylated immunotoxin comprising a) loading a solution containing the non-glycosylated immunotoxin onto a hydrophobic interaction column; b) obtaining a first non-glycosylated immunotoxin containing eluant from the hydrophobic interaction column; c) loading the non-glycosylated immunotoxin containing eluant from step (b) onto an anion exchange column; d) obtaining a second non-glycosylated immunotoxin containing eluant from the anion exchange column by eluting the non-glycosylated immunotoxin with a sodium borate solution; e) diluting the concentration of sodium borate in the second non-glycosylated immunotoxin containing eluant from step (d) to about 50 mM or less; f) concentrating the diluted non-glycosylated immunotoxin containing eluant from step (e) over an anion exchange column; and g) obtaining a purified non-glycosylated immunotoxin from the anion exchange column.

H. sapiens(66%)	...706 DVTLHADAIHRGGGQIIPTARR...858
M. musculus(3'mRNA)	..... DVTLHADAIHRGGGQIIPTARR...
R. norvegicus(66%)	...706 DVTLHADAIHRGGGQIIPTARR...858
C. griseus(CHO, 67%)	...706 DVTLHADAIHRGGGQIIPTARR...858
D. melanogaster(67%)	...692 DVTLHADAIHRGGGQIIPTTRR...848
C. elegans (67%)	...700 DVTLHADAIHRGGGQIIPTARR...852
S. pombe (79%)	...690 DVVLHADAIHRGGGQIIPTARR...842
P. pastoris (88%)	...690 DVTLHADAIHRGGGQVIPTMKR...842
S. cerevisiae	...690 DVTLHADAIHRGGGQIIPTMRR...842




```
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                                                R
                                            cGc cGc GGA
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   D   V   T   L   H   A   D   A   I   H   R   G   G   G   Q   V   I   P   T   M   K   R
690                                        700                                     710
```
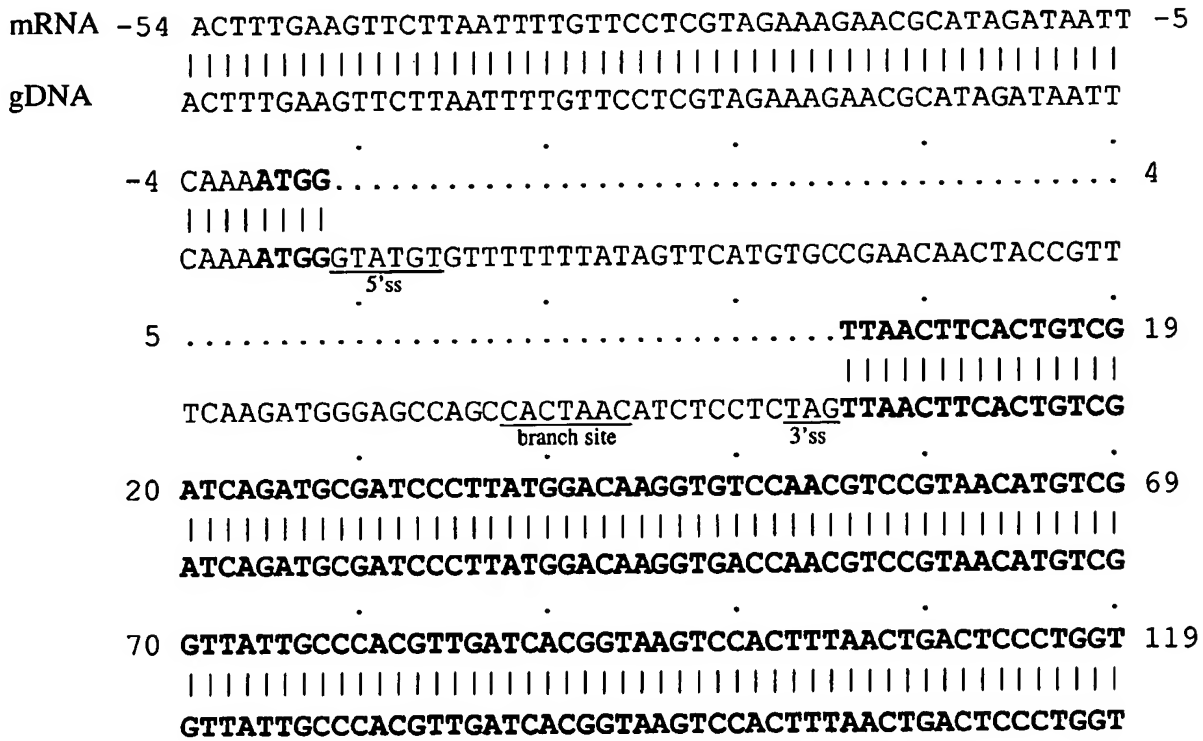

FIG. 1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gDNA ACTTTGAAGTTCTTAATTTTGTTCCTCGTAGAAAGAACGCATAGATAATT
-4 CAAAATGG 4
CAAAATGGGTATGTGTTTTTTTATAGTTCATGTGCCGAACAACTACCGTT
5'ss
5 TTAACTTCACTGTCG 19
TCAAGATGGGAGCCAGCCACTAACATCTCCTCTAGTTAACTTCACTGTCG
branch site
3'ss
20 ATCAGATGCGATCCCTTATGGACAAGGTGTCCAACGTCCGTAACATGTCG 69
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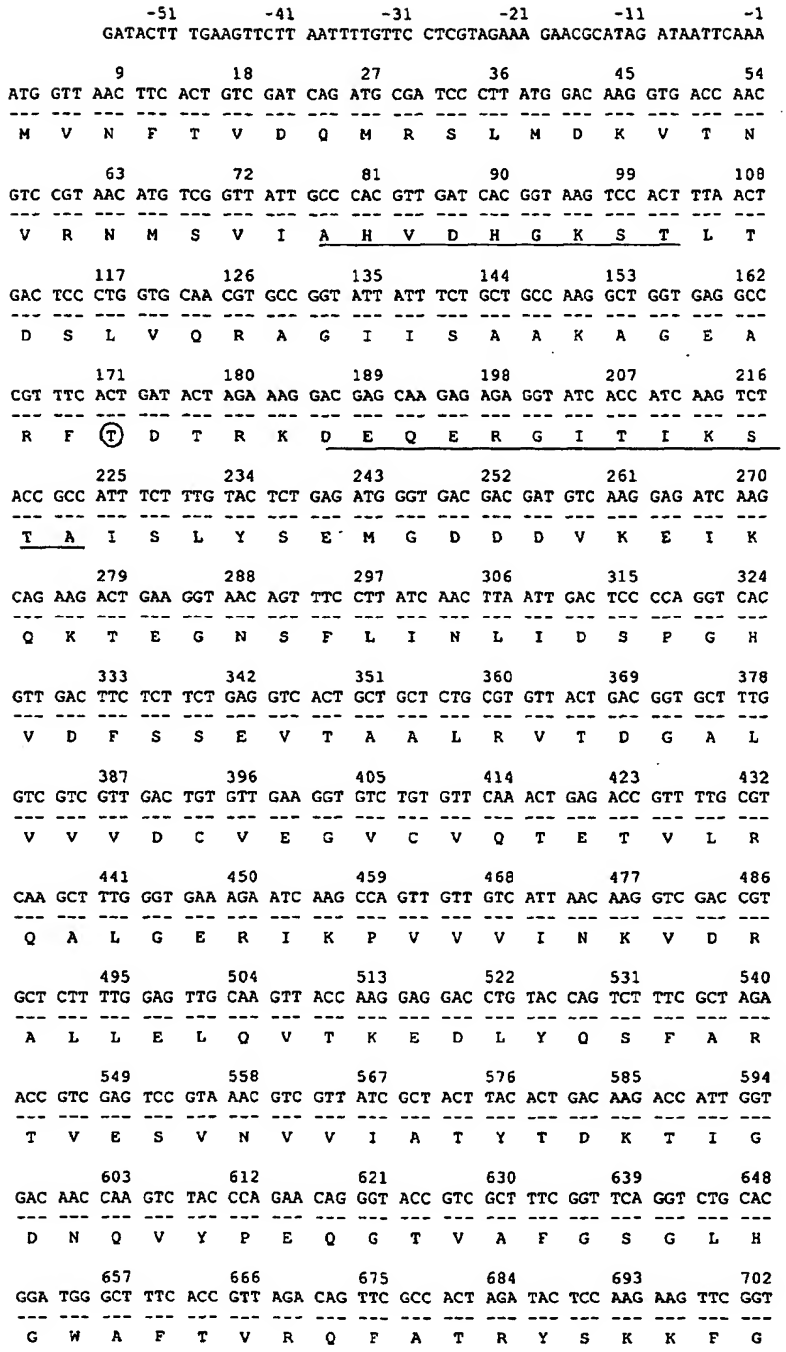
GTTATTGCCCACGTTGATCACGGTAAGTCCACTTTAACTGACTCCCTGGT

FIG. 2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9 18 27 36 45 54
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M V N F T V D Q M R S L M D K V T N
63 72 81 90 99 108
GTC CGT AAC ATG TCG GTT ATT GCC CAC GTT GAT CAC GGT AAG TCC ACT TTA ACT
V R N M S V I A H V D H G K S T L T
117 126 135 144 153 162
GAC TCC CTG GTG CAA CGT GCC GGT ATT ATT TCT GCT GCC AAG GCT GGT GAG GCC
D S L V Q R A G I I S A A K A G E A
171 180 189 198 207 216
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R F T D T R K D E Q E R G I T I K S
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T A I S L Y S E M G D D D V K E I K
279 288 297 306 315 324
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Q K T E G N S F L I N L I D S P G H
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T V E S V N V V I A T Y T D K T I G
603 612 621 630 639 648
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D N Q V Y P E Q G T V A F G S G L H
657 666 675 684 693 702
GGA TGG GCT TTC ACC GTT AGA CAG TTC GCC ACT AGA TAC TCC AAG AAG TTC GGT
G W A F T V R Q F A T R Y S K K F G

FIG. 3A

```
        711         720         729         738         747         756
GTT GAC AGA ATC AAG ATG ATG GAG CGT CTG TGG GGA GAC TCT TAC TTC AAC CCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   D   R   I   K   M   M   E   R   L   W   G   D   S   Y   F   N   P

        765         774         783         792         801         810
AAG ACC AAG AAA TGG ACC AAC AAG GAC AAG GAC GCC GCT GGA AAG CCT TTG GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   T   K   K   W   T   N   K   D   K   D   A   A   G   K   P   L   E

        819         828         837         846         855         864
CGT GCC TTC AAC ATG TTC GTT TTG GAC CCT ATC TTC CGT CTG TTT GCT GCC ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   A   F   N   M   F   V   L   D   P   I   F   R   L   F   A   A   I

        873         882         891         900         909         918
ATG AAC TTC AAG AAG GAT GAA ATT CCA GTT CTG TTG GAG AAA TTG GAG ATC AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   N   F   K   K   D   E   I   P   V   L   L   E   K   L   E   I   N

        927         936         945         954         963         972
CTG AAG CGT GAG GAG AAG GAG TTG GAG GGT AAG GCT CTT TTG AAG GTT GTC ATG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   K   R   E   E   K   E   L   E   G   K   A   L   L   K   V   V   M

        981         990         999        1008        1017        1026
AGA AAG TTC TTG CCA GCT GCC GAC GCT TTG TTG GAG ATG ATT GTT CTT CAC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   K   F   L   P   A   A   D   A   L   L   E   M   I   V   L   H   L

       1035        1044        1053        1062        1071        1080
CCA TCT CCA GTC ACC GCT CAA GCT TAC AGA GCC GAG ACT TTG TAC GAA GGT CCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   S   P   V   T   A   Q   A   Y   R   A   E   T   L   Y   E   G   P

       1089        1098        1107        1116        1125        1134
TCT GAT GAC CAA TTC TGC ATT GGT ATC AGA GAG TGT GAC CCT AAG GCT GAG CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   D   D   Q   F   C   I   G   I   R   E   C   D   P   K   A   E   L

       1143        1152        1161        1170        1179        1188
ATG GTT TAC ATT TCC AAG ATG GTG CCA ACC TCC GAC AAA GGT AGA TTC TAC GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   V   Y   I   S   K   M   V   P   T   S   D   K   G   R   F   Y   A

       1197        1206        1215        1224        1233        1242
TTC GGT CGT GTT TTC TCC GGT ACT GTT AAG TCC GGT CAA AAG GTC AGA ATC CAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   G   R   V   F   S   G   T   V   K   S   G   Q   K   V   R   I   Q

       1251        1260        1269        1278        1287        1296
GGT CCT AAC TAC GTT CCA GGT AAG AAG GAG GAC TTG TTC ATC AAG GCT GTT CAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   P   N   Y   V   P   G   K   K   E   D   L   F   I   K   A   V   Q

       1305        1314        1323        1332        1341        1350
AGA ACT GTT TTG ATG ATG GGA AGA ACC GTC GAG CCT ATT GAC GAT GTC CCA GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   T   V   L   M   M   G   R   T   V   E   P   I   D   D   V   P   A

       1359        1368        1377        1386        1395        1404
GGT AAC ATT CTG GGT ATT GTG GGT ATC GAC CAG TTC TTG CTG AAG TCT GGT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   N   I   L   G   I   V   G   I   D   Q   F   L   L   K   S   G   T

                          ┌─Δ5'EF-2
       1413        1422   │    1431        1440        1449        1458
CTT ACT ACC AAC GAA GCC GCT CAC AAC ATG AAG GTG ATG AAA TTC TCT GTC TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   T   T   N   E   A   A   H   N   M   K   V   M   K   F   S   V   S
```

FIG. 3B

```
      1467        1476        1485        1494        1503        1512
CCA GTT GTG CAA GTT GCC GTT GAG GTC AAG AAC GCT AAT GAT CTG CCC AAG TTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   V   V   Q   V   A   V   E   V   K   N   A   N   D   L   P   K   L

      1521        1530        1539        1548        1557        1566
GTT GAG GGT CTG AAG CGT TTG TCC AAG TCT GAC CCA TGT GTT TTA ACC TAC ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   E   G   L   K   R   L   S   K   S   D   P   C   V   L   T   Y   I

      1575        1584        1593        1602        1611        1620
TCC GAG TCT GGT GAG CAC ATT GTT GCT GGT ACT GGT GAG CTG CAC TTG GAA ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   E   S   G   E   H   I   V   A   G   T   G   E   L   H   L   E   I

      1629        1638        1647        1656        1665        1674
TGT TTG CAA GAT CTG CAA GAC GAC CAC GCT GGT GTC CCT CTG AAG ATT TCT CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   L   Q   D   L   Q   D   D   H   A   G   V   P   L   K   I   S   P

      1683        1692        1701        1710        1719        1728
CCA GTT GTT ACC TAC CGT GAG ACT GTC ACT AAC GAA TCT TCC ATG ACT GCC CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   V   V   T   Y   R   E   T   V   T   N   E   S   S   M   T   A   L

      1737        1746        1755        1764        1773        1782
TCC AAG TCT CAG AAC AAG CAT AAC AGA ATT TAC CTG AAG GCT CAA CCA ATT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   K   S   Q   N   K   H   N   R   I   Y   L   K   A   Q   P   I   D

      1791        1800        1809        1818        1827        1836
GAG GAA TTG TCT TTG GCT ATC GAA GAA GGT AAG GTT CAC CCA AGA GAC GAC TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   E   L   S   L   A   I   E   E   G   K   V   H   P   R   D   D   F

      1845        1854        1863        1872        1881        1890
AAA GCC AGA GCC AGA ATC ATG GCT GAT GAA TAC GGT TGG GAC GTC ACT GAT GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   A   R   A   R   I   M   A   D   E   Y   G   W   D   V   T   D   A

      1899        1908        1917        1926        1935        1944
AGA AAG ATC TGG TGT TTC GGT CCA GAC GGT ACT GGT GCC AAC TTA GTT GTT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   K   I   W   C   F   G   P   D   G   T   G   A   N   L   V   V   D

      1953        1962        1971        1980        1989        1998
CAG TCT AAG GCT GTC CAA TAC TTG CAC GAG ATC AAG GAC TCT GTT GTT GCC GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   S   K   A   V   Q   Y   L   H   E   I   K   D   S   V   V   A   G

      2007        2016        2025        2034        2043        2052
TTC CAA TTG GCT ACC AAG GAA GGT CCA ATT TTG GGA GAA AAC ATG AGA TCC GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   Q   L   A   T   K   E   G   P   I   L   G   E   N   M   R   S   V

      2061        2070        2079        2088        2097        2106
AGA GTC AAC ATC TTG GAT GTT ACC CTG CAC GCC GAT GCT ATC CAC AGA GGT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   V   N   I   L   D   V   T   L   H   A   D   A   I   H   R   G   G

      2115        2124        2133        2142        2151        2160
GGA CAA GTC ATT CCA ACC ATG AAG AGA GTT ACC TAC GCC GCC TTC CTG TTG GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   Q   V   I   P   T   M   K   R   V   T   Y   A   A   F   L   L   A

      2169        2178        2187        2196        2205        2214
GAG CCA GCT ATC CAG GAG CCT ATC TTC TTG GTG GAG ATC CAA TGT CCA GAG AAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   P   A   I   Q   E   P   I   F   L   V   E   I   Q   C   P   E   N
```

FIG. 3C

```
      2223        2232        2241        2250        2259        2268
GCC ATT GGT GGT ATC TAC TCT GTT TTG AAC AAG AAG AGA GGT CAA GTT ATC TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   I   G   G   I   Y   S   V   L   N   K   K   R   G   Q   V   I   S

      2277        2286        2295        2304        2313        2322
GAG GAA CAA AGA CCA GGT ACC CCA TTG TTC ACT GTC AAA GCT TAC TTG CCA GTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   E   Q   R   P   G   T   P   L   F   T   V   K   A   Y   L   P   V

      2331        2340        2349        2358        2367        2376
AAC GAG TCA TTC GGT TTC ACC GGT GAA CTG AGA CAA GCT ACC GCT GGT CAA GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   E   S   F   G   F   T   G   E   L   R   Q   A   T   A   G   Q   A

      2385        2394        2403        2412        2421        2430
TTC CCA CAG ATG GTG TTC GAC CAC TGG GCC AAC ATG AAT GGT AAC CCA TTG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   P   Q   M   V   F   D   H   W   A   N   M   N   G   N   P   L   D

      2439        2448        2457        2466        2475        2484
CCA GCC TCC AAG GTC GGT GAG ATT GTT CTT GCT GCC AGA AAG AGA CAG GGT ATG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   A   S   K   V   G   E   I   V   L   A   A   R   K   R   Q   G   M

      2493        2502        2511        2520        2529        2538
AAG GAG AAC GTT CCT GGT TAT GAA GAG TAC TAC GAC AAG TTG TAA GCT TAA TGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   E   N   V   P   G   Y   E   E   Y   Y   D   K   L

      2547        2556        2565        2574        2583        2592
TTC ATT AAC TTA TTT GTG TCG TTC GTA TGT CTA TTT ACG TAC TTA ATT CAG TGT
    A5'EF-2

      2601
ATT GTT GTT 3'
```

FIG. 3D

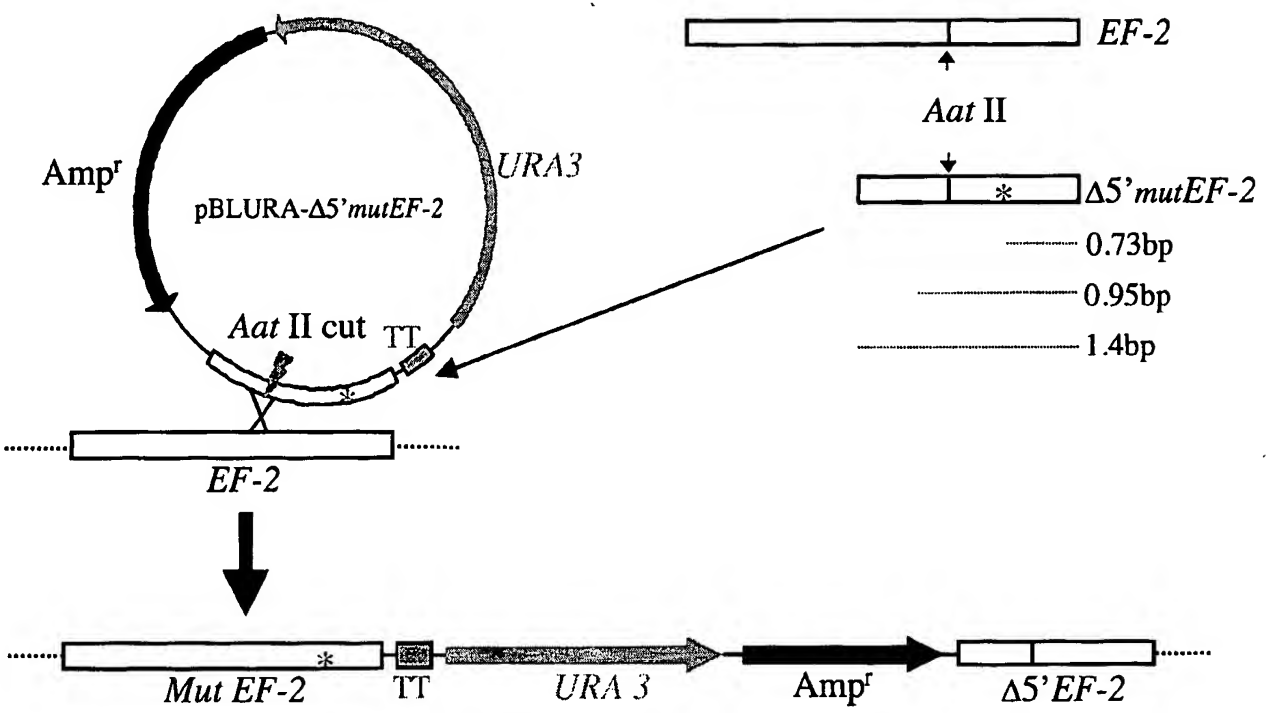

EF-2
Aat II
Δ5'mutEF-2
0.73bp
0.95bp
1.4bp
Amp^r
URA3
pBLURA-Δ5'mutEF-2
Aat II cut
TT
EF-2
Mut EF-2
TT
URA 3
Amp^r
Δ5'EF-2

FIG. 4

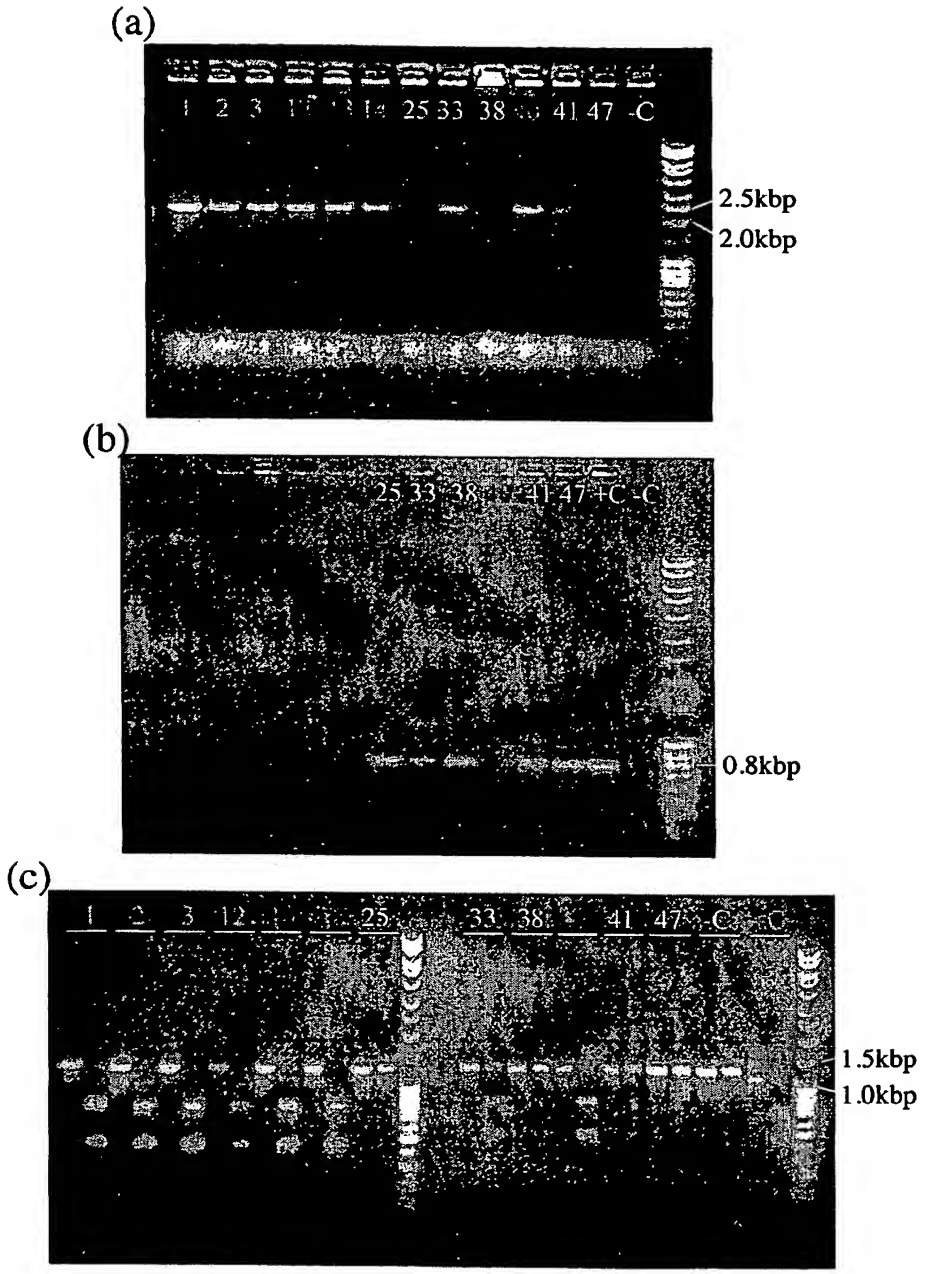

(a)
1 2 3 25 33 38 41 47 -C
2.5kbp
2.0kbp
(b)
25 33 38 41 47 +C -C
0.8kbp
(c)
1 2 3 12 25 33 38 41 47 -C -C
1.5kbp
1.0kbp

FIG. 5

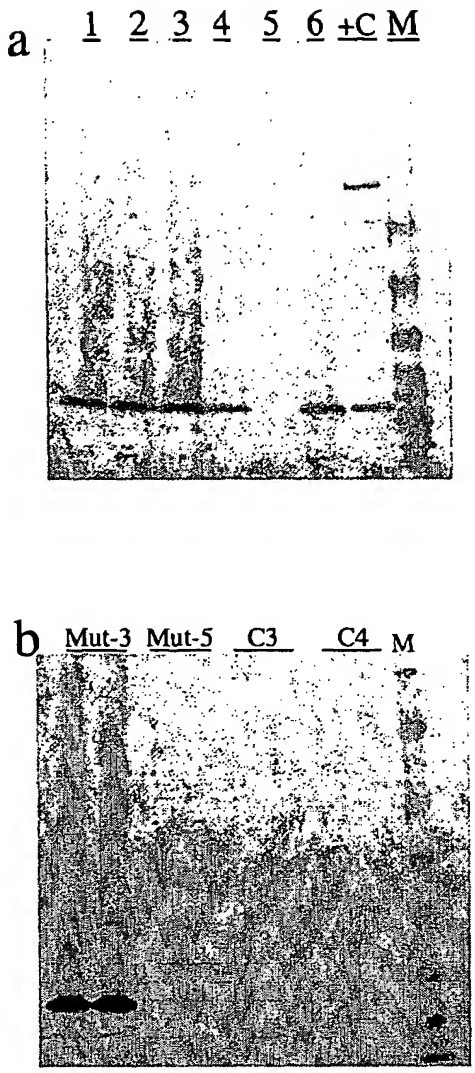


FIG. 6

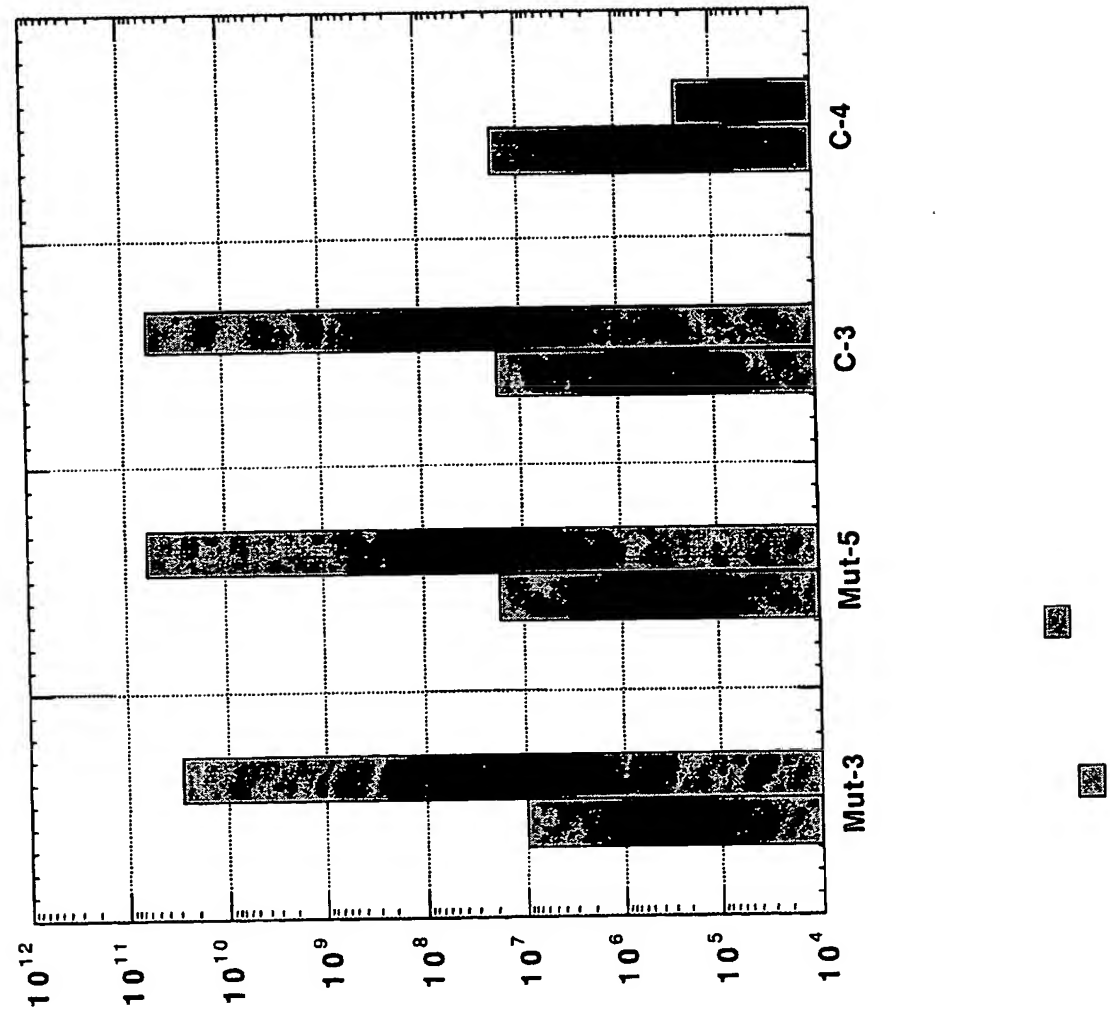


FIG. 7

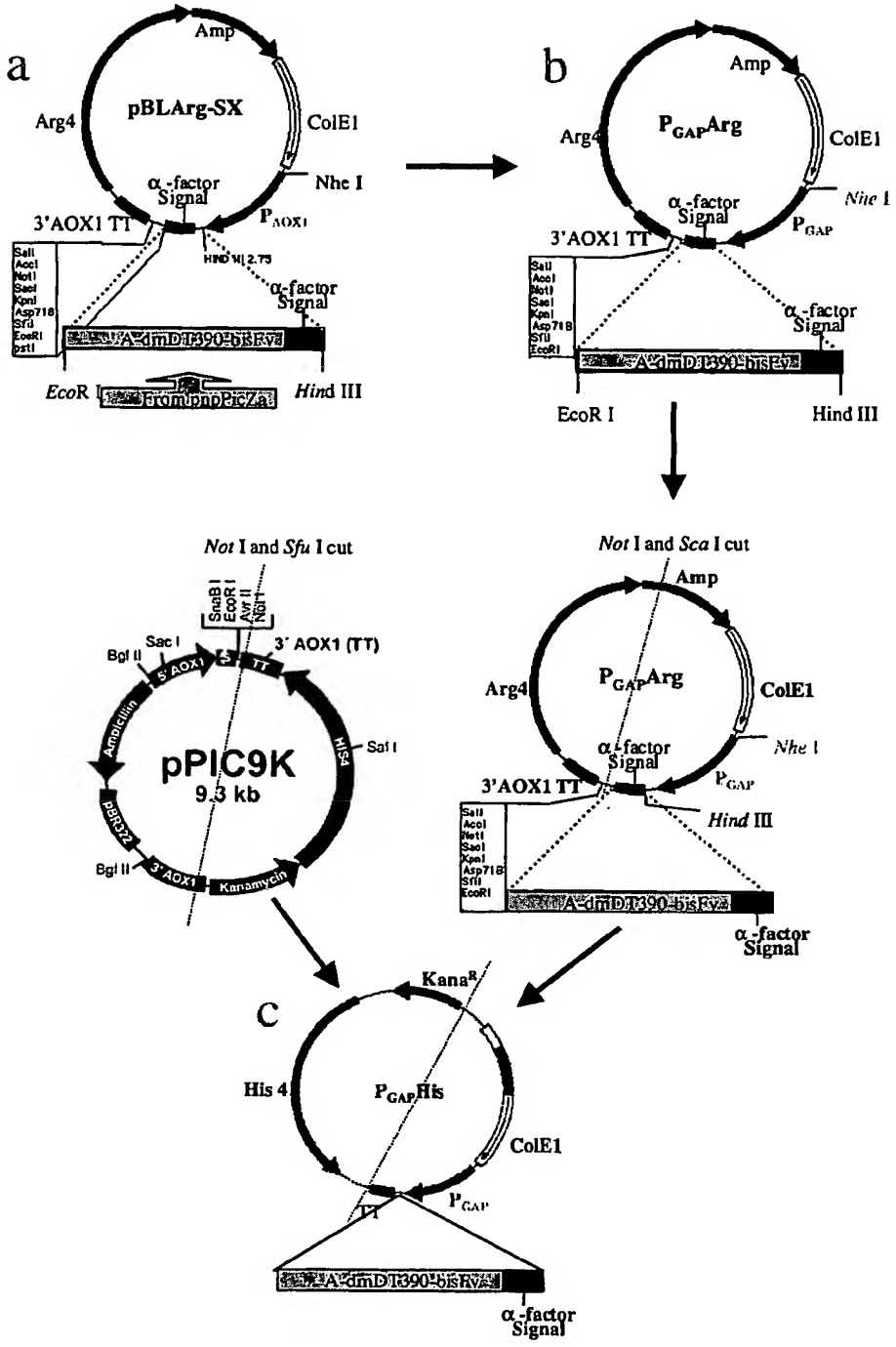
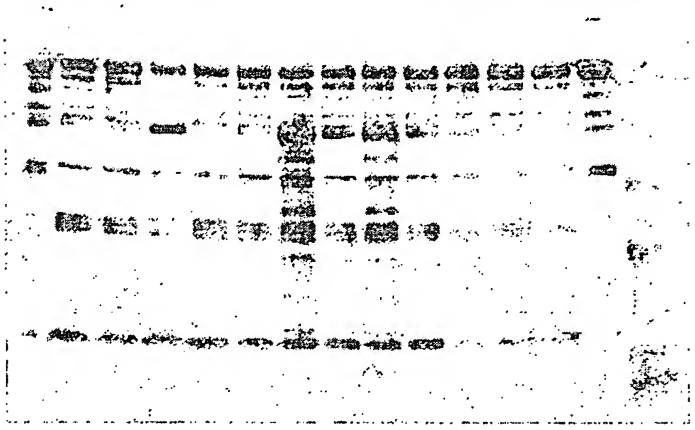

a
Amp
pBLArg-SX
Arg4
ColE1
α-factor Signal
Nhe I
3'AOX1 TT
P_{AOX1}
Hind III 2.75
α-factor Signal
EcoR I
Hind III
b
Amp
P_{GAP}Arg
Arg4
ColE1
Nhe I
α-factor Signal
P_{GAP}
3'AOX1 TT
α-factor Signal
EcoR I
Hind III
Not I and Sfu I cut
Not I and Sca I cut
pPIC9K
9.3 kb
Amp
Arg4
ColE1
Nhe I
3'AOX1 TT
Hind III
α-factor Signal
c
Kana^R
His 4
P_{GAP}His
ColE1
P_{GAP}
α-factor Signal

FIG. 8

a



b

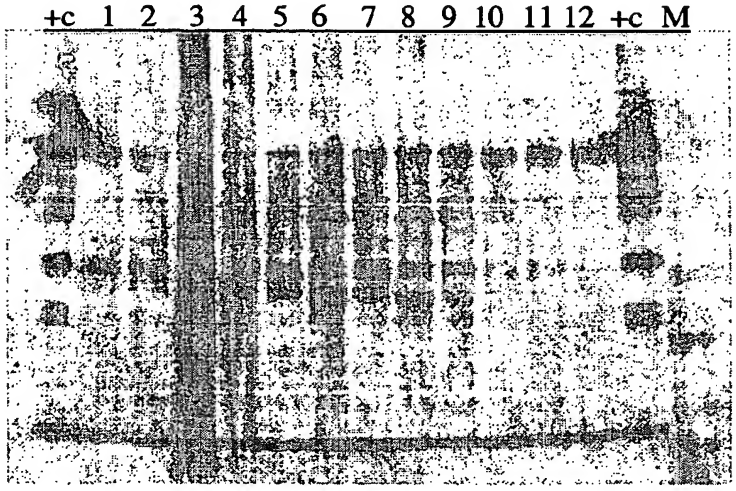


FIG. 9

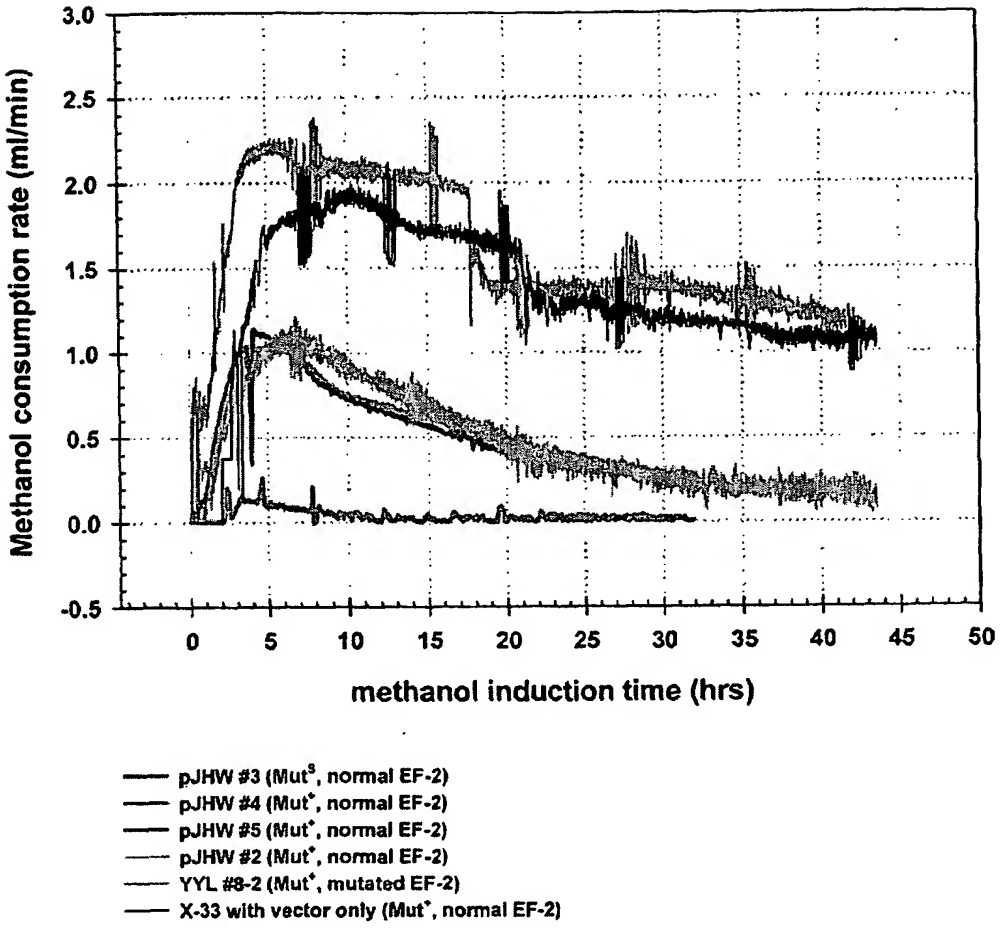

Methanol consumption rate (ml/min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
5
10
15
20
25
30
35
40
45
50
methanol induction time (hrs)
pJHW #3 (Mut^S, normal EF-2)
pJHW #4 (Mut$^+$, normal EF-2)
pJHW #5 (Mut$^+$, normal EF-2)
pJHW #2 (Mut$^+$, normal EF-2)
YYL #8-2 (Mut$^+$, mutated EF-2)
X-33 with vector only (Mut$^+$, normal EF-2)

FIG. 10

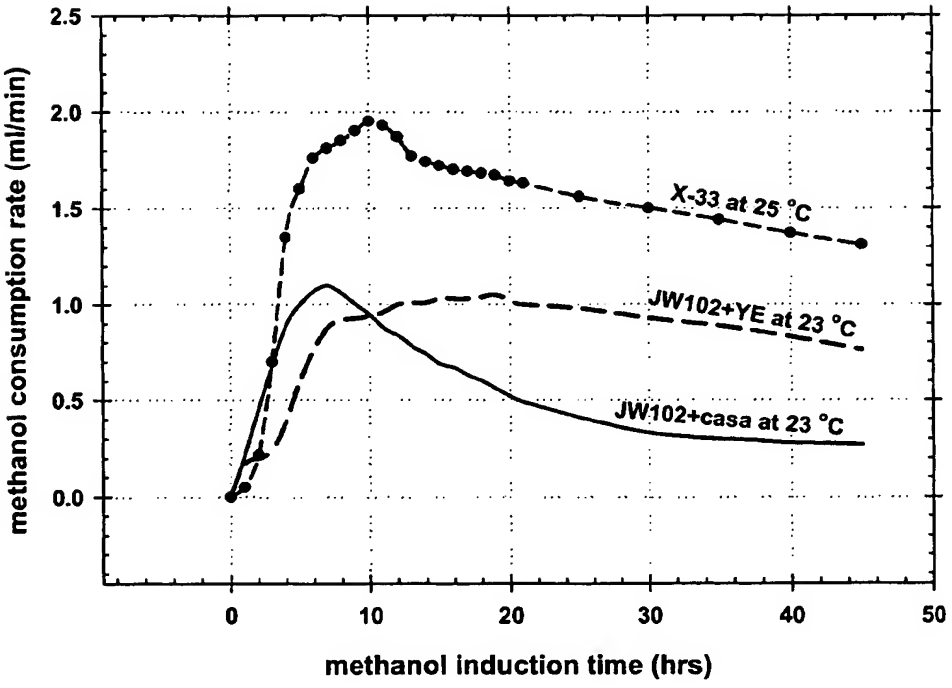

methanol consumption rate (ml/min)
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
methanol induction time (hrs)
X-33 at 25 °C
JW102+YE at 23 °C
JW102+casa at 23 °C

FIG. 11

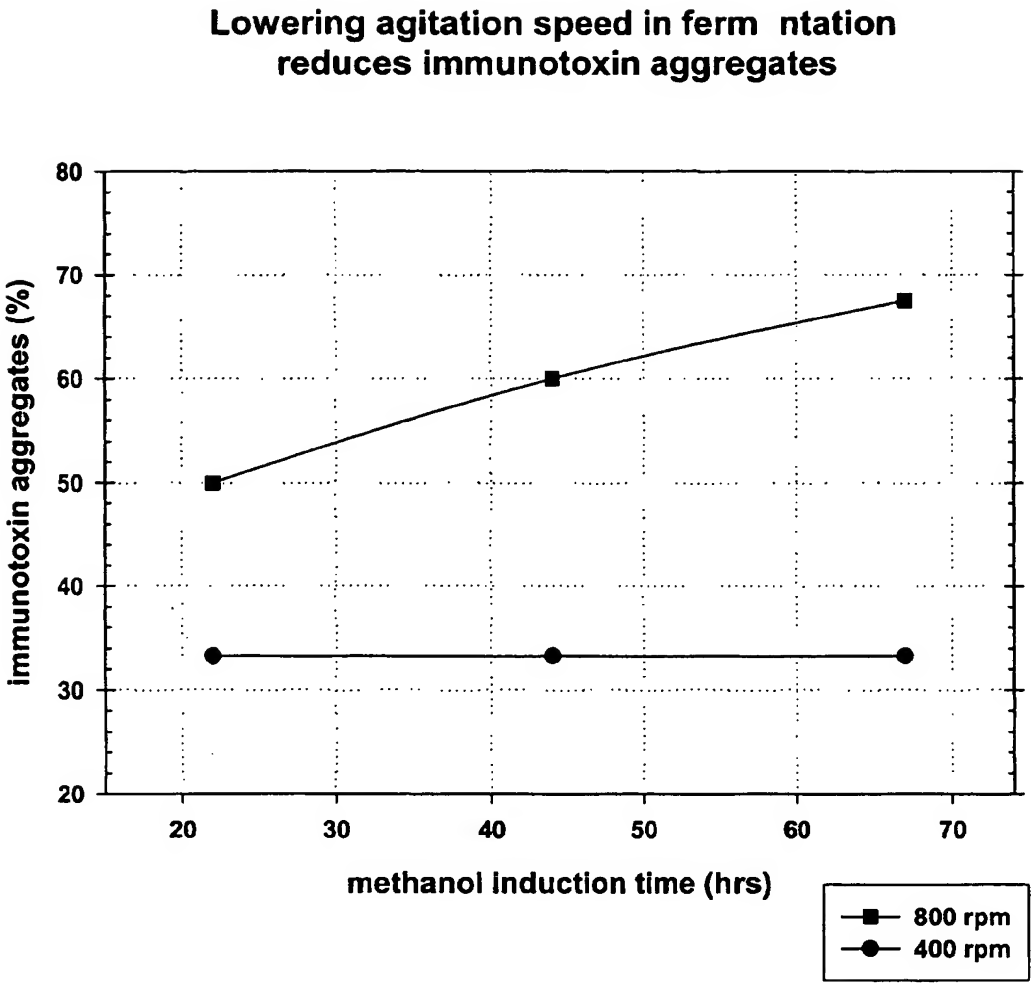

Lowering agitation speed in ferm ntation
reduces immunotoxin aggregates
immunotoxin aggregates (%)
80
70
60
50
40
30
20
20
30
40
50
60
70
methanol induction time (hrs)
800 rpm
400 rpm

Fig. 12

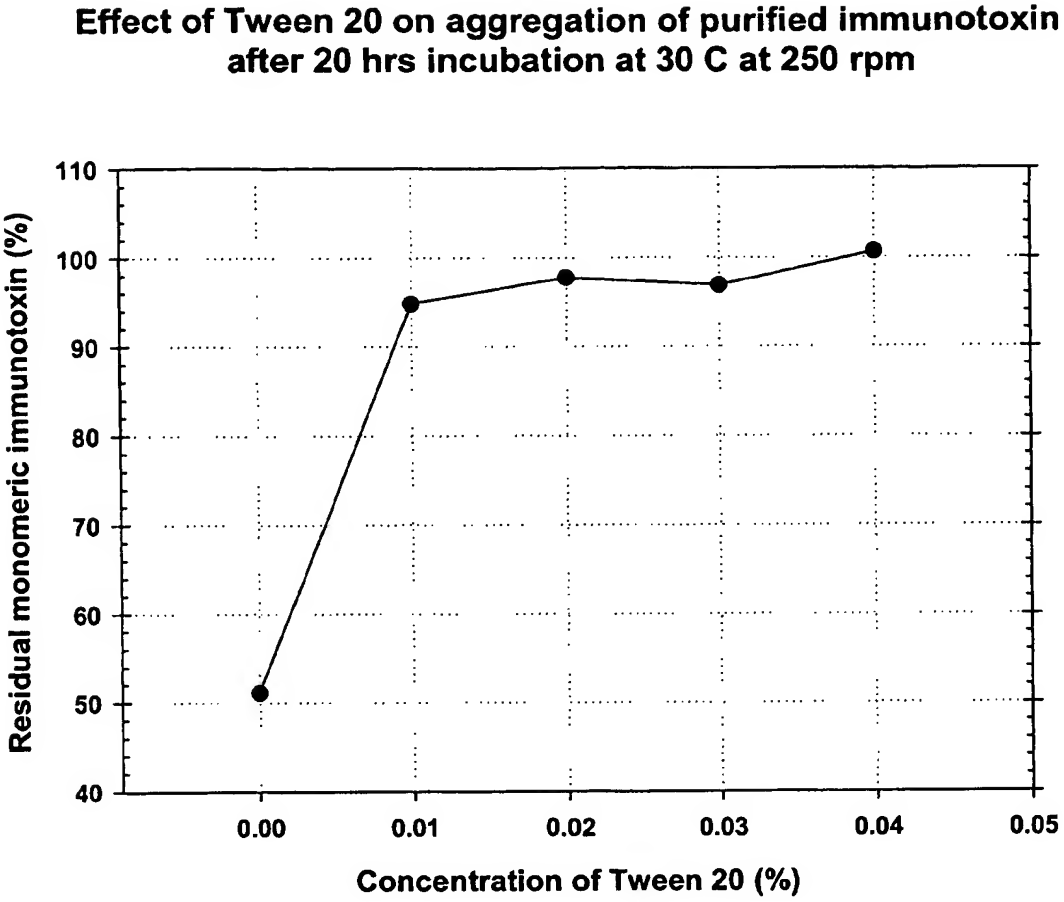

Effect of Tween 20 on aggregation of purified immunotoxin after 20 hrs incubation at 30 C at 250 rpm
Residual monomeric immunotoxin (%)
110
100
90
80
70
60
50
40
0.00
0.01
0.02
0.03
0.04
0.05
Concentration of Tween 20 (%)

Fig. 13

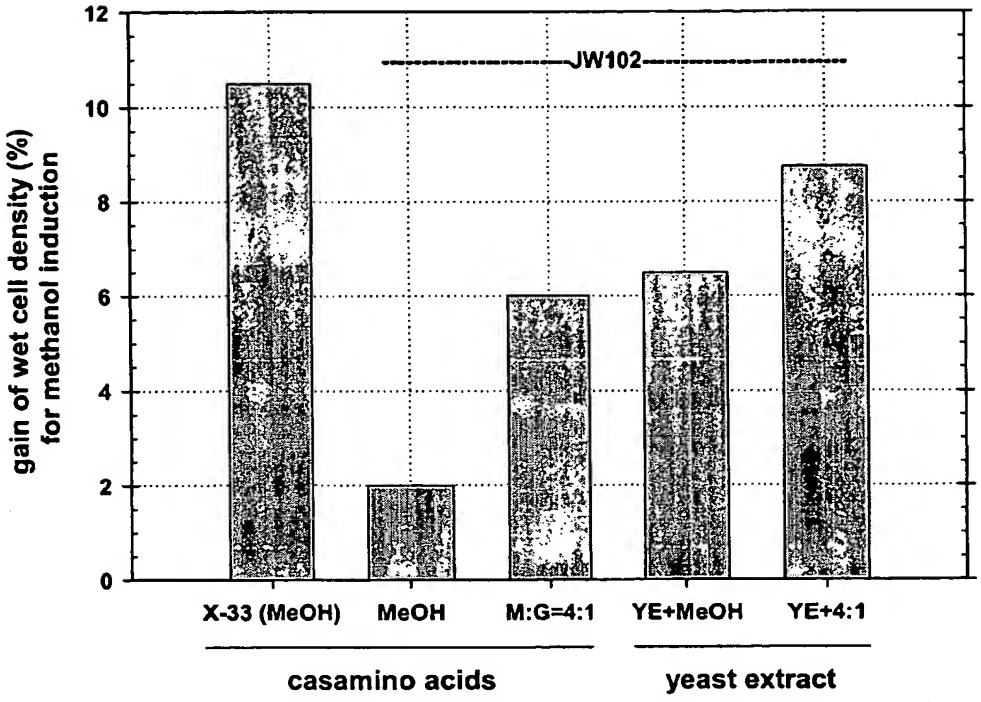

gain of wet cell density (%)
for methanol induction
12
10
8
6
4
2
0
JW102
X-33 (MeOH)
MeOH
M:G=4:1
YE+MeOH
YE+4:1
casamino acids
yeast extract

FIG. 14

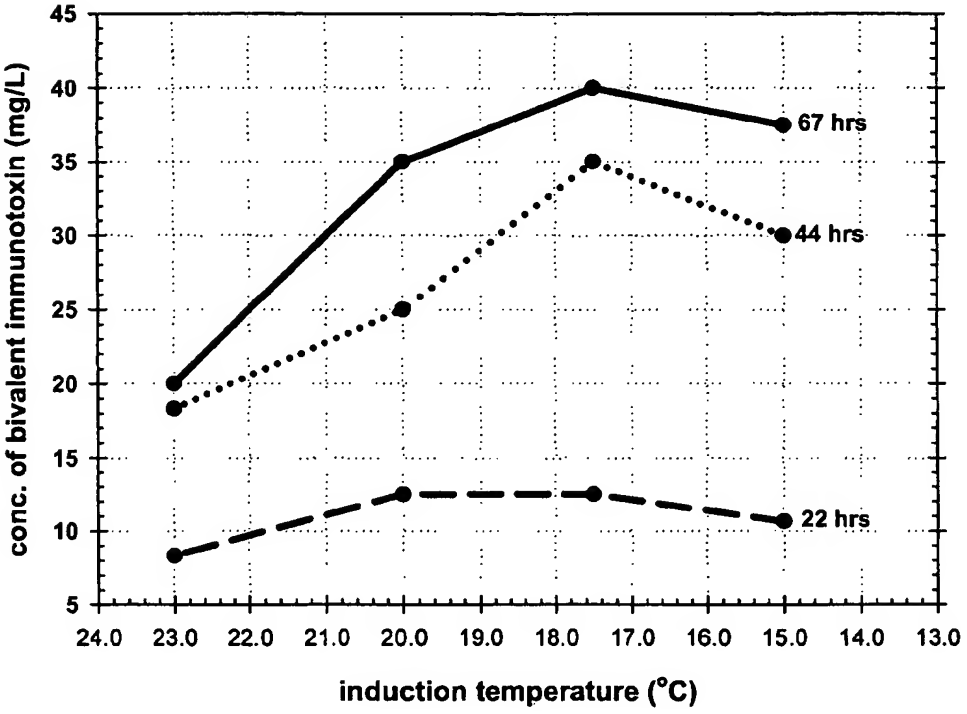

45
40
35
30
25
20
15
10
5
conc. of bivalent immunotoxin (mg/L)
67 hrs
44 hrs
22 hrs
24.0
23.0
22.0
21.0
20.0
19.0
18.0
17.0
16.0
15.0
14.0
13.0
induction temperature (°C)

FIG. 15A

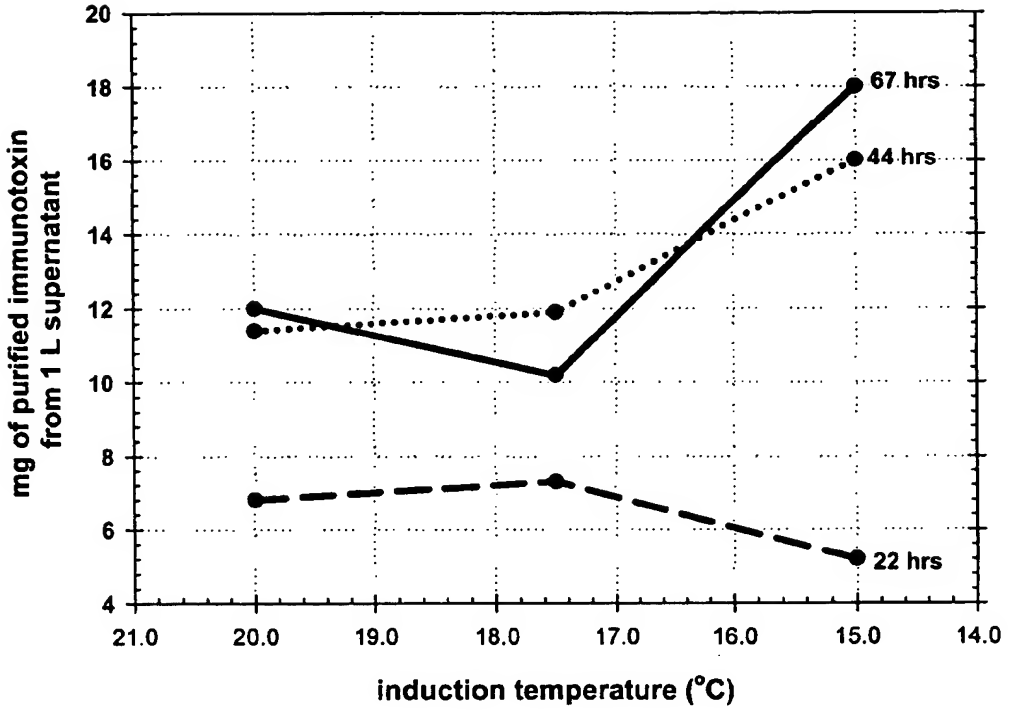

20
18
16
14
12
10
8
6
4
mg of purified immunotoxin from 1 L supernatant
67 hrs
44 hrs
22 hrs
21.0
20.0
19.0
18.0
17.0
16.0
15.0
14.0
induction temperature (°C)

FIG. 15B

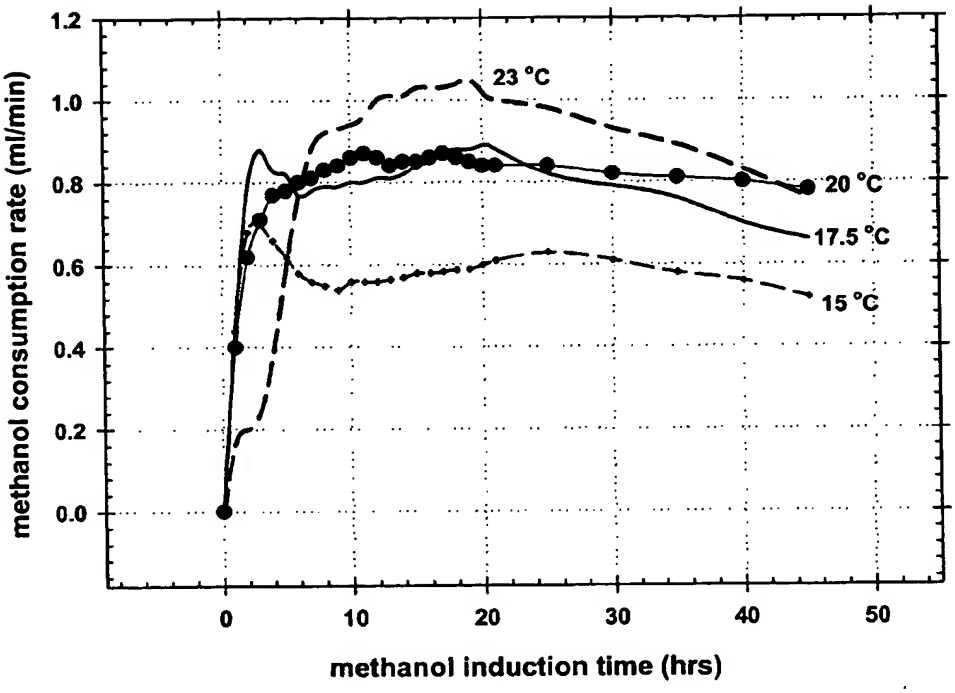

1.2
1.0
0.8
0.6
0.4
0.2
0.0
methanol consumption rate (ml/min)
23 °C
20 °C
17.5 °C
15 °C
0
10
20
30
40
50
methanol induction time (hrs)

FIG. 15C

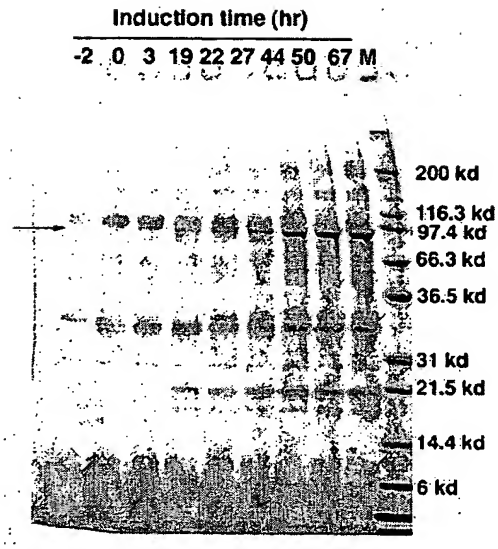

Induction time (hr)
-2 0 3 19 22 27 44 50 67 M
200 kd
116.3 kd
97.4 kd
66.3 kd
36.5 kd
31 kd
21.5 kd
14.4 kd
6 kd

FIG. 16

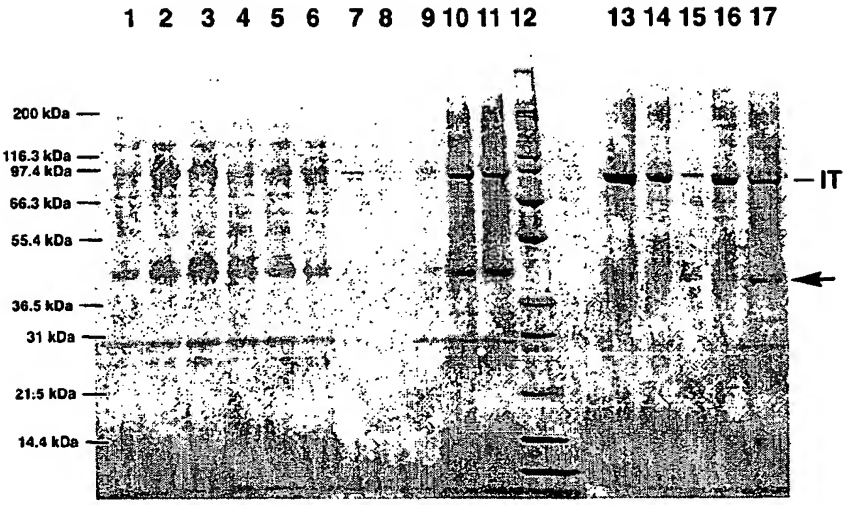

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31 kDa
21.5 kDa
14.4 kDa
IT

FIG. 17

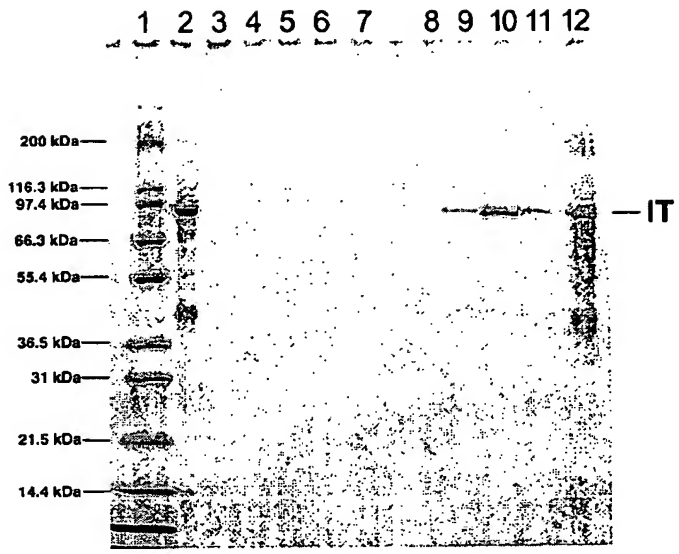
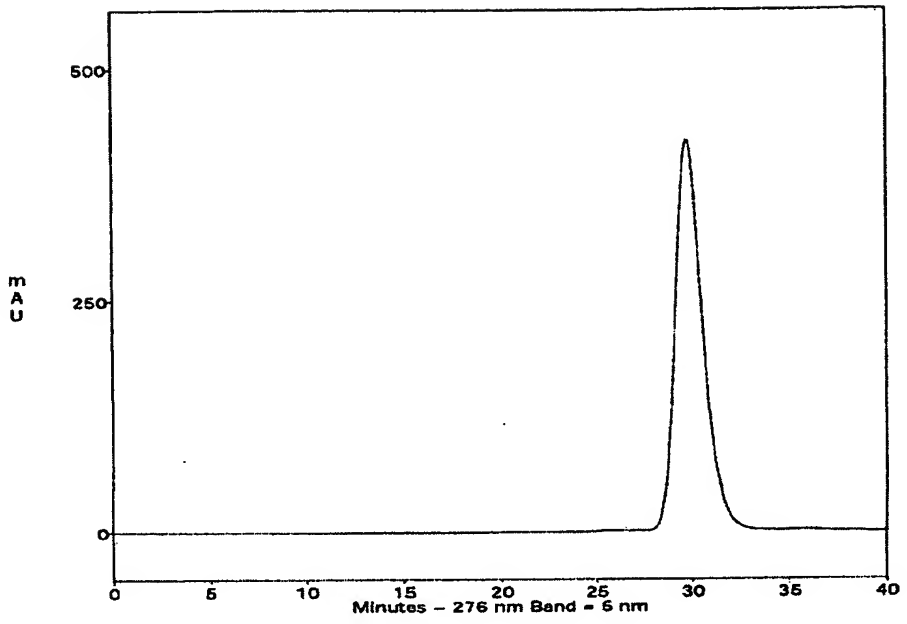

1 2 3 4 5 6 7 8 9 10 11 12
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31 kDa
21.5 kDa
14.4 kDa
IT

FIG. 18

A



B

FIG. 19

Ala Gly Ala Asp Asp Val Val Asp Ser Ser Lys Ser Phe Val Met Glu
1 5 10 15
Asn Phe Ala Ser Tyr His Gly Thr Lys Pro Gly Tyr Val Asp Ser Ile
20 25 30
Gln Lys Gly Ile Gln Lys Pro Lys Ser Gly Thr Gln Gly Asn Tyr Asp
35 40 45
Asp Asp Trp Lys Gly Phe Tyr Ser Thr Asp Asn Lys Tyr Asp Ala Ala
50 55 60
Gly Tyr Ser Val Asp Asn Glu Asn Pro Leu Ser Gly Lys Ala Gly Gly
65 70 75 80
Val Val Lys Val Thr Tyr Pro Gly Leu Thr Lys Val Leu Ala Leu Lys
85 90 95
Val Asp Asn Ala Glu Thr Ile Lys Lys Glu Leu Gly Leu Ser Leu Thr
100 105 110
Glu Pro Leu Met Glu Gln Val Gly Thr Glu Glu Phe Ile Lys Arg Phe
115 120 125
Gly Asp Gly Ala Ser Arg Val Val Leu Ser Leu Pro Phe Ala Glu Gly
130 135 140
Ser Ser Ser Val Glu Tyr Ile Asn Asn Trp Glu Gln Ala Lys Ala Leu
145 150 155 160
Ser Val Glu Leu Glu Ile Asn Phe Glu Thr Arg Gly Lys Arg Gly Gln
165 170 175
Asp Ala Met Tyr Glu Tyr Met Ala Gln Ala Cys Ala Gly Asn Arg Val
180 185 190
Arg Arg Ser Val Gly Ser Ser Leu Ser Cys Ile Asn Leu Asp Trp Asp
195 200 205
Val Ile Arg Asp Lys Thr Lys Thr Lys Ile Glu Ser Leu Lys Glu His
210 215 220
Gly Pro Ile Lys Asn Lys Met Ser Glu Ser Pro Ala Lys Thr Val Ser
225 230 235 240
Glu Glu Lys Ala Lys Gln Tyr Leu Glu Glu Phe His Gln Thr Ala Leu
245 250 255
Glu His Pro Glu Leu Ser Glu Leu Lys Thr Val Thr Gly Thr Asn Pro
260 265 270
Val Phe Ala Gly Ala Asn Tyr Ala Ala Trp Ala Val Asn Val Ala Gln
275 280 285
Val Ile Asp Ser Glu Thr Ala Asp Asn Leu Glu Lys Thr Thr Ala Ala
290 295 300
Leu Ser Ile Leu Pro Gly Ile Gly Ser Val Met Gly Ile Ala Asp Gly
305 310 315 320
Ala Val His His Asn Thr Glu Glu Ile Val Ala Gln Ser Ile Ala Leu
325 330 335
Ser Ser Leu Met Val Ala Gln Ala Ile Pro Leu Val Gly Glu Leu Val
340 345 350
Asp Ile Gly Phe Ala Ala Tyr Asn Phe Val Glu Ser Ile Ile Asn Leu
355 360 365
Phe Gln Val Val His Asn Ser Tyr Asn Arg Pro Ala Tyr Ser Pro Gly
370 375 380
His Lys Thr Gln Pro Phe Leu Pro Trp Asp Ile Gln Met Thr Gln Thr
385 390 395 400
Thr Ser Ser Leu Ser Ala Ser Leu Gly Asp Arg Val Thr Ile Ser Cys
405 410 415

FIG. 20A

Arg Ala Ser Gln Asp Ile Arg Asn Tyr Leu Asn Trp Tyr Gln Gln Lys
420 425 430
Pro Asp Gly Thr Val Lys Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His
435 440 445
Ser Gly Val Pro Ser Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
450 455 460
Ser Leu Thr Ile Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe
465 470 475 480
Cys Gln Gln Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys
485 490 495
Leu Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
500 505 510
Gly Gly Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
515 520 525
Pro Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe
530 535 540
Thr Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
545 550 555 560
Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr Asn
565 570 575
Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
580 585 590
Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp Ser Ala Val
595 600 605
Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser Asp Trp Tyr Phe
610 615 620
Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
625 630 635 640
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met
645 650 655
Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly Asp Arg Val Thr
660 665 670
Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg Asn Tyr Leu Asn Trp Tyr
675 680 685
Gln Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile Tyr Tyr Thr Ser
690 695 700
Arg Leu His Ser Gly Val Pro Ser Lys Phe Ser Gly Ser Gly Ser Gly
705 710 715 720
Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Gln Glu Asp Ile Ala
725 730 735
Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly
740 745 750
Gly Thr Lys Leu Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly
755 760 765
Ser Gly Gly Gly Gly Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu
770 775 780
Leu Val Lys Pro Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly
785 790 795 800
Tyr Ser Phe Thr Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly
805 810 815
Lys Asn Leu Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser
820 825 830

FIG. 20B

Thr Tyr Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys
835 840 845
Ser Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
850 855 860
Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser Asp
865 870 875 880
Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Thr Leu Thr Val Phe Ser
885 890 896

FIG. 20C

SEQUENCE LISTING

<110> NEVILLE, David
WOO, Jung-Hee
LIU, Yuan-Yi

<120> METHODS FOR EXPRESSION AND PURIFICATION
OF IMMUNOTOXINS

<130> 14028.0295

<140> Unassigned
<141> 2003-08-01

<160> 35

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 22
<212> PRT
<213> H. sapiens

<400> 1
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1 5 10 15
Ile Pro Thr Ala Arg Arg
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<210> 2
<211> 22
<212> PRT
<213> M. musculus

<400> 2
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
1 5 10 15
Ile Pro Thr Ala Arg Arg
20

<210> 3
<211> 22
<212> PRT
<213> R. norvegicus

<400> 3
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
1 5 10 15
Ile Pro Thr Ala Arg Arg
20

<210> 4
<211> 22
<212> PRT
<213> C. griseus

<400> 4
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
1 5 10 15
Ile Pro Thr Ala Arg Arg
20

<210> 5
<211> 22
<212> PRT
<213> D. melanogaster

<400> 5
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
 1   5   10   15
Ile Pro Thr Thr Arg Arg
            20

<210> 6
<211> 22
<212> PRT
<213> C. elegans

<400> 6
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
 1   5   10   15
Ile Pro Thr Ala Arg Arg
            20

<210> 7
<211> 22
<212> PRT
<213> S. pombe

<400> 7
Asp Val Val Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
 1   5   10   15
Ile Pro Thr Ala Arg Arg
            20

<210> 8
<211> 22
<212> PRT
<213> P. pastoris

<400> 8
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Val
 1   5   10   15
Ile Pro Thr Met Lys Arg
            20

<210> 9
<211> 22
<212> PRT
<213> S. cerevisiae

<400> 9
Asp Val Thr Leu His Ala Asp Ala Ile His Arg Gly Gly Gly Gln Ile
 1   5   10   15
Ile Pro Thr Met Arg Arg
            20

<210> 10
<211> 66
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
      synthetic construct

<400> 10
gatgttaccc tgcacgccga tgctatccac cgccgcggag gacaagtcat tccaaccatg   60
aagaga   66

<210> 11
<211> 223
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 11
actttgaagt tcttaatttt gttcctcgta gaaagaacgc atagataatt caaaatggca 60
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tcgatcagat gcgatccctt atggacaagg tgtccaacgt ccgtaacatg tcggttattg 180
cccacgttga tcacggtaag tccactttaa ctgactccct ggt 223

<210> 12
<211> 250
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 12
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taacatctcc tctagttaac ttcactgtcg atcagatgcg atcccttatg gacaaggtga 180
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actccctggt 250

<210> 13
<211> 2601
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 13
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caacgtgccg gtattatttc tgctgccaag gctggtgagg cccgtttcac tgatactaga 180
aaggacgagc aagagagagg tatcaccatc aagtctaccg ccatttcttt gtactctgag 240
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aacttaattg actccccagg tcacgttgac ttctcttctg aggtcactgc tgctctgcgt 360
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ttaattcagt gtattgttgt t 2601

<210> 14
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 14
Ala His Val Asp His Gly Lys Ser Thr
1 5

<210> 15
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 15
Asp Glu Gln Glu Arg Gly Ile Thr Ile Lys Ser Thr Ala
1 5 10

<210> 16
<211> 896
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 16
Ala Gly Ala Asp Asp Val Val Asp Ser Ser Lys Ser Phe Val Met Glu
1 5 10 15
Asn Phe Ala Ser Tyr His Gly Thr Lys Pro Gly Tyr Val Asp Ser Ile
20 25 30
Gln Lys Gly Ile Gln Lys Pro Lys Ser Gly Thr Gln Gly Asn Tyr Asp
35 40 45
Asp Asp Trp Lys Gly Phe Tyr Ser Thr Asp Asn Lys Tyr Asp Ala Ala
50 55 60
Gly Tyr Ser Val Asp Asn Glu Asn Pro Leu Ser Gly Lys Ala Gly Gly
65 70 75 80
Val Val Lys Val Thr Tyr Pro Gly Leu Thr Lys Val Leu Ala Leu Lys
85 90 95
Val Asp Asn Ala Glu Thr Ile Lys Lys Glu Leu Gly Leu Ser Leu Thr
100 105 110
Glu Pro Leu Met Glu Gln Val Gly Thr Glu Glu Phe Ile Lys Arg Phe
115 120 125
Gly Asp Gly Ala Ser Arg Val Val Leu Ser Leu Pro Phe Ala Glu Gly
130 135 140
Ser Ser Ser Val Glu Tyr Ile Asn Asn Trp Glu Gln Ala Lys Ala Leu

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145                 150                 155                 160
Ser Val Glu Leu Glu Ile Asn Phe Glu Thr Arg Gly Lys Arg Gly Gln
                165                 170                 175
Asp Ala Met Tyr Glu Tyr Met Ala Gln Ala Cys Ala Gly Asn Arg Val
            180                 185                 190
Arg Arg Ser Val Gly Ser Ser Leu Ser Cys Ile Asn Leu Asp Trp Asp
        195                 200                 205
Val Ile Arg Asp Lys Thr Lys Thr Lys Ile Glu Ser Leu Lys Glu His
    210                 215                 220
Gly Pro Ile Lys Asn Lys Met Ser Glu Ser Pro Ala Lys Thr Val Ser
225                 230                 235                 240
Glu Glu Lys Ala Lys Gln Tyr Leu Glu Glu Phe His Gln Thr Ala Leu
                245                 250                 255
Glu His Pro Glu Leu Ser Glu Leu Lys Thr Val Thr Gly Thr Asn Pro
            260                 265                 270
Val Phe Ala Gly Ala Asn Tyr Ala Ala Trp Ala Val Asn Val Ala Gln
        275                 280                 285
Val Ile Asp Ser Glu Thr Ala Asp Asn Leu Glu Lys Thr Thr Ala Ala
    290                 295                 300
Leu Ser Ile Leu Pro Gly Ile Gly Ser Val Met Gly Ile Ala Asp Gly
305                 310                 315                 320
Ala Val His His Asn Thr Glu Glu Ile Val Ala Gln Ser Ile Ala Leu
                325                 330                 335
Ser Ser Leu Met Val Ala Gln Ala Ile Pro Leu Val Gly Glu Leu Val
            340                 345                 350
Asp Ile Gly Phe Ala Ala Tyr Asn Phe Val Glu Ser Ile Ile Asn Leu
        355                 360                 365
Phe Gln Val Val His Asn Ser Tyr Asn Arg Pro Ala Tyr Ser Pro Gly
    370                 375                 380
His Lys Thr Gln Pro Phe Leu Pro Trp Asp Ile Gln Met Thr Gln Thr
385                 390                 395                 400
Thr Ser Ser Leu Ser Ala Ser Leu Gly Asp Arg Val Thr Ile Ser Cys
                405                 410                 415
Arg Ala Ser Gln Asp Ile Arg Asn Tyr Leu Asn Trp Tyr Gln Gln Lys
            420                 425                 430
Pro Asp Gly Thr Val Lys Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His
        435                 440                 445
Ser Gly Val Pro Ser Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
    450                 455                 460
Ser Leu Thr Ile Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe
465                 470                 475                 480
Cys Gln Gln Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys
                485                 490                 495
Leu Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
            500                 505                 510
Gly Gly Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
        515                 520                 525
Pro Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe
    530                 535                 540
Thr Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
545                 550                 555                 560
Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr Asn
                565                 570                 575
Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
            580                 585                 590
Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp Ser Ala Val
        595                 600                 605
Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser Asp Trp Tyr Phe
    610                 615                 620
Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
625                 630                 635                 640
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met
                645                 650                 655
Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly Asp Arg Val Thr
            660                 665                 670
Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg Asn Tyr Leu Asn Trp Tyr
        675                 680                 685
Gln Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile Tyr Tyr Thr Ser
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    690                 695                 700
Arg Leu His Ser Gly Val Pro Ser Lys Phe Ser Gly Ser Gly Ser Gly
705                 710                 715                 720
Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Gln Glu Asp Ile Ala
                725                 730                 735
Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly
            740                 745                 750
Gly Thr Lys Leu Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly
        755                 760                 765
Ser Gly Gly Gly Gly Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu
    770                 775                 780
Leu Val Lys Pro Gly Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly
785                 790                 795                 800
Tyr Ser Phe Thr Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly
                805                 810                 815
Lys Asn Leu Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser
            820                 825                 830
Thr Tyr Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys
        835                 840                 845
Ser Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
    850                 855                 860
Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser Asp
865                 870                 875                 880
Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Thr Leu Thr Val Phe Ser
                885                 890                 895
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<210> 17
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<221> misc_feature
<222> 15
<223> S = G or C

<400> 17
ggggsggggs ggggs 15

<210> 18
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<221> misc_feature
<222> 4,8,12,16
<223> s = g or c

<400> 18
gggsgggsgg gsgggs 16

<210> 19
<211> 3
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<221> VARIANT

<222> 2
<223> Xaa= any amino acid

<221> VARIANT
<222> 3
<223> Xaa = s or t

<400> 19
Asn Xaa Xaa
 1

<210> 20
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 20
ttggttattg accaaactaa ggctgtccaa 30

<210> 21
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 21
acctctcttc ttgtttaaga cggagtagat 30

<210> 22
<211> 39
<212> DNA
<213> Artificia Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 22
cttgcttttg cggccgcttt ttttttttt ttttttttt 39

<210> 23
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 23
gataagaatg cggccgccat ttcttggtct ttgggttgaa g 41

<210> 24
<211> 42
<212> DNA
<213> Artificia Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 24
gataagaatg cggccgccaa cttagttgtt gaccagtcta ag 42

<210> 25
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 25
atagctagca ctttgaagtt cttaattttg ttcctc 36

<210> 26
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 26
ataagaatgc ggccgcaagt taatgaaaca ttaagcttac aac 43

<210> 27
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 27
gaatgacttg tcctccacc 19

<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 28
gaatgacttg tcctccgcgg 20

<210> 29
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 29
caactagcta gcgctcacaa catgaaggtc atgaaattc 39

<210> 30
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 30
agaaccgtcg agcctattga cgat 24

<210> 31
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 31
ccctgcacgc cgatgctatc cacagaagag gaggacaagt cattccaacc atgaag 56

<210> 32
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 32
gccgatgcta tccacagaag a 21

<210> 33
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 33
gccgatgcta tccaccgccg c 21

<210> 34
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<400> 34
tctcttcttg ttcaaaacag agtagatacc 30

<210> 35
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; note =
synthetic construct

<221> misc_feature
<222> 7,15
<223> n = g,a, c or t(u)

<400> 35
gtatgtncac taacntag 18